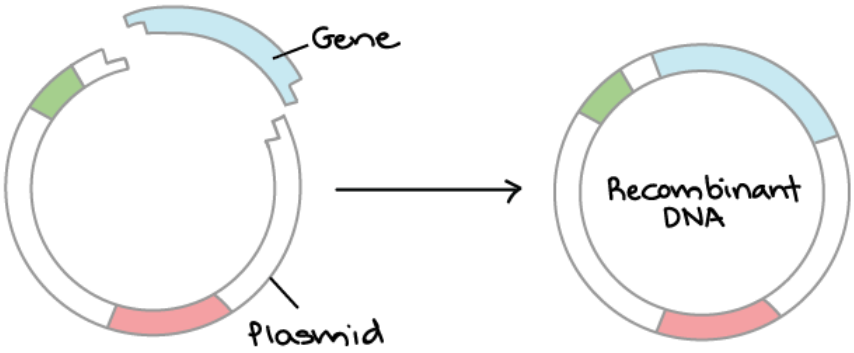
AIM OF THE PROJECT

The aim of the project is to use gene therapy to kill cancer cells *in vitro*. To achieve this goal the gene encoding human papillomavirus E2 protein will be introduced into human cervical cancer cells (HeLa cell line).

PROJECT SCHEDULE

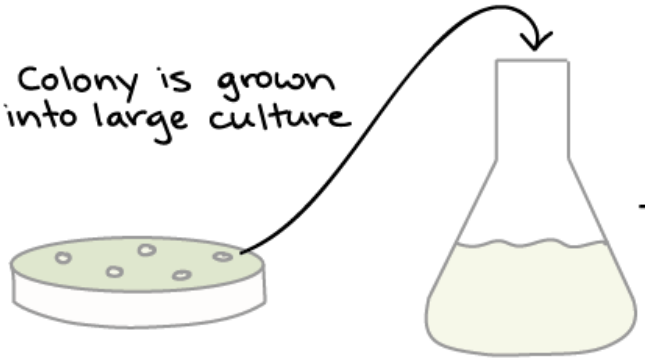
DAY 1 – PLASMID ISOLATION

In order to obtain a vector expressing the E2 protein for gene therapy, the *E2* gene was inserted (cloned) into an expression plasmid – pE2.



[https://www.khanacademy.org]

Then the recombinant plasmid was placed in *E. coli* bacterial cells in the process of transformation. Bacteria were grown on selective culture media and the ones carrying the correct version of the vector were cultured on a large scale in liquid medium – THESE STEPS WERE ALREADY DONE FOR YOU.



[https://www.khanacademy.org]

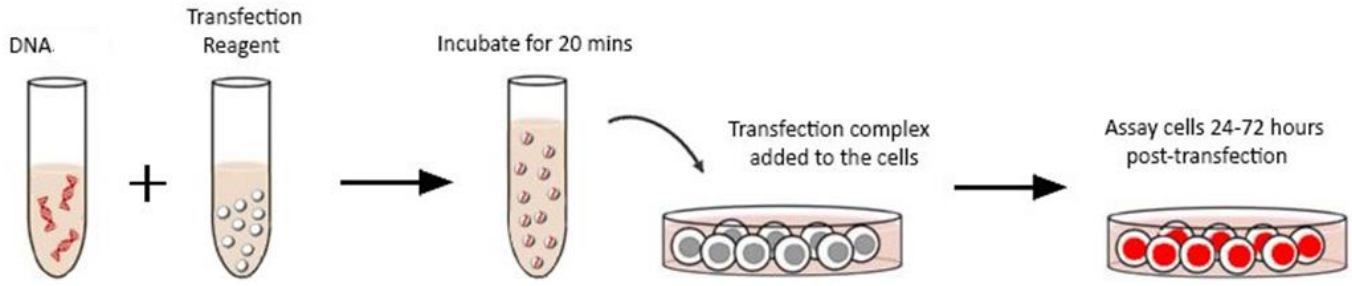
On day 1 you will use the cultured bacteria to isolate the pE2 vector. During the procedure you will destroy bacterial cells by lysis with the use of different types of buffers. This way their genetic material (including the pE2 vector) will be released. In the next steps you will purify the plasmid DNA in a series of centrifugation steps.

DAY 2, PART 1 – GENE THERAPY OF CANCER CELLS

On day 2, the pE2 vector will be used for gene therapy of cancer cells. In this approach you will use the purified plasmid from day 1 and apply it to the *in vitro* culture of HeLa cells.

The method of transferring a vector inside eukaryotic cells is called transfection. To aid this process DNA is combined with special agents which make it easier for the cells to perform endocytosis of the vector. In this case you will use a solution of liposomes. Liposomes will be mixed with plasmid DNA, which results in forming complexes of the two. Then the solution containing DNA-liposome complexes will be mixed with the cells on culture plates.

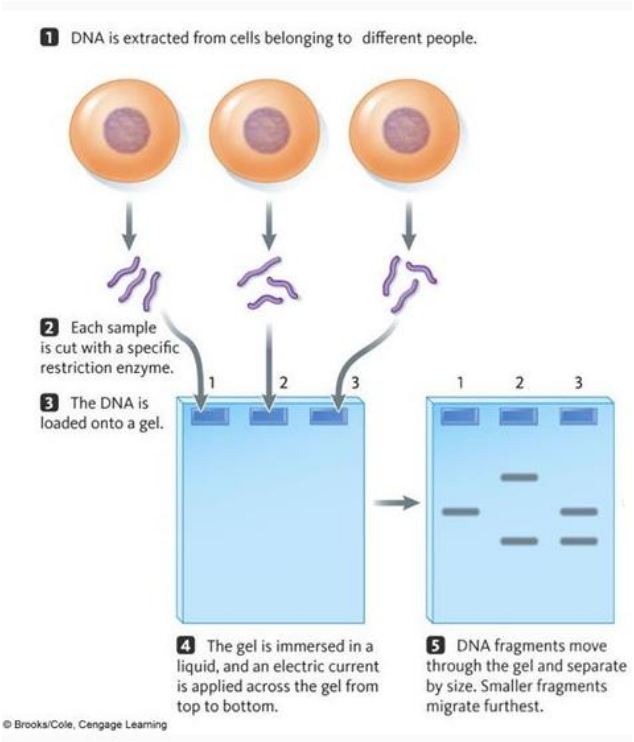
The effects of the adopted gene therapy will be evaluated 48 hours after transfection by staining and microscopic evaluation.



[https://www.abmgood.com]

DAY 2, PART 2 – HPV DIAGNOSTICS

In this part of the project you will perform an analysis, which allows determining the genotype of HPV derived from different individuals. This way you will be able to state if any of the analyzed individuals has increased risk of developing cervical cancer due to HPV infection.



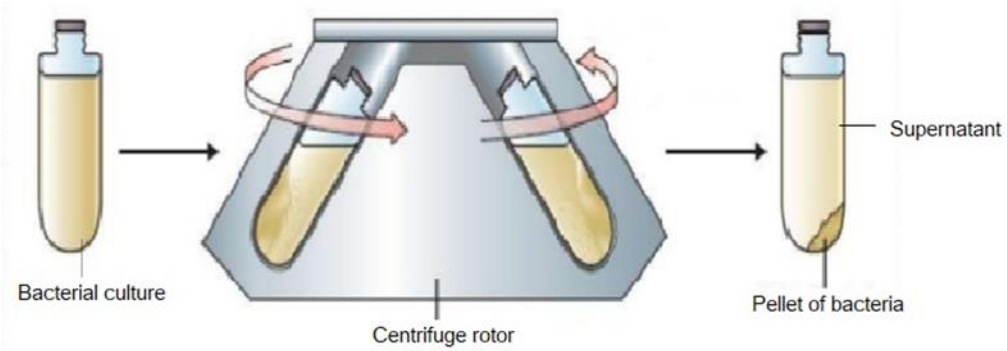
HPV genotyping will be based on the use of RFLP. In this assay the products of PCR reaction (in which viral DNA, isolated from different individuals, was used as a template) will be cut with restriction enzymes. The pieces formed after this step will be separated in gel electrophoresis. On the basis of the pattern created by the pieces in an agarose gel the genotype of the HPV will be determined.

PROTOCOL FOR PLASMID ISOLATION

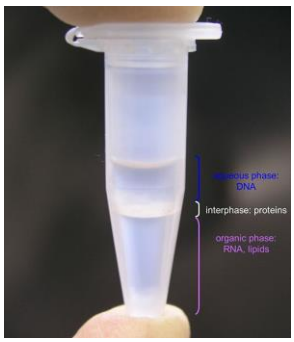
Materials required:

Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0), Solution II (0,2 N NaOH, 1% SDS), Solution III (5M potassium acetate, acetic acid), 99.6% ethanol, H_2O, pipettes, eppendorf tubes

1. Transfer 1 mL of bacterial culture into a 1.5mL tube.
2. Centrifuge (5,000 rpm, 5 min).



3. Carefully discard the supernatant.
4. Add 50 µl of ice-cold Solution I to the bacterial pellet. Mix by pippeting up and down.
5. Add 100 µl of Solution II to the suspension. Mix by gently inverting the tubes.
6. Incubate on ice for 5 min.
7. Add 75 µl ice-cold Solution III and vortex for 10 seconds.
8. Incubate on ice for 5 min.
9. Centrifuge (12,500; 5 min).
10. Transfer the supernatant into a new Eppendorf tube.
11. Add 100 µl of phenol:chloroform:isoamylalcohol (25:24:1) to the supernatant.
12. Mix by vortexing for 10 sec.
13. Centrifuge (12,500 rpm; 5 min).
14. Transfer 200 µl of the upper phase (transparent) of the solution to a fresh tube (see phases in picture on the right).
15. Add 500 µl of 99.6% ethanol. Incubate in RT for 2 min.
16. Centrifuge (12,500 rpm; 5 min).
17. Remove supernatant. The pellet is plasmid DNA.
18. Air-dry the pellet for about 10 min.
19. Dissolve DNA in 20µl of H2O.
20. Incubate for 10 min at -20°C.
21. Measure the concentration of DNA with a spectrophotometer.



[https://openwetware.org

PROTCOL FOR GENE THERAPY OF EUKARYOTIC CELLS *IN VITRO*

PART I Transfection

https://www.sigmaaldrich.com

Materials required:

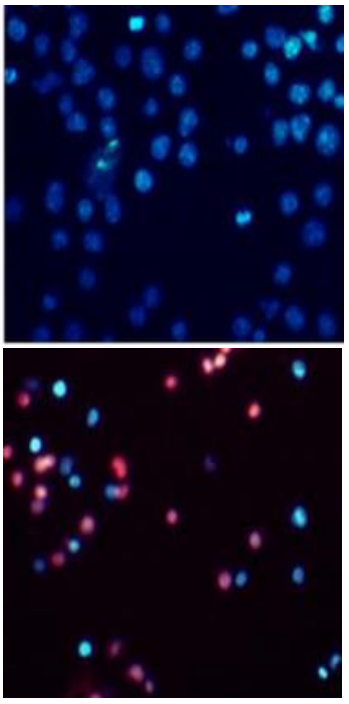
serum-free RPMI medium, Turbofect transfection reagent (liposomes), plasmid DNA, $0.3x10^6$ HeLa cells seeded on a 6-well plates, pipettes, eppendorf tubes

pE2 vector concentration: …….

volume of pE2 which holds 1µg of DNA …….

1. Transfer 25 µL of serum-free RPMI medium into an Eppendorf tube.
2. Add 1µg of plasmid DNA into the medium.
3. Briefly vortex the transfection reagent (5 sec).
4. Add 2µL of the transfection reagent to the tube holding medium and DNA.
5. Mix immediately by vortexing (5 sec).
6. Incubate the transfection solution 15 minutes at room temperature.
7. After the incubation time take out 2 plates from the incubator, label them with C (control) and T (transfection).
8. Add transfection solution to the plate labelled with T.
9. Gently rock the plate immediately after adding the transfection solution, to achieve even distribution of the complexes.
10. Put the plates back in the CO_2 incubator.
11. Analyze transgene expression after 48 hours.

PART II Cell staining



Materials required:

propidium iodide[0.1 g/ml] and Hoechst 33342 [0.125 g/ml] solution, pipettes

1. Take out plates transfected 48hrs ago from the incubator.
2. Add 5 µl of propidium iodide/Hoechst 33342 solution into the cells transfected with pE2 vector and control cells.
3. Put the plates in the CO_2 incubator and incubate 10 minutes.
4. Analyze cell using a fluorescence microscope.

PROTOCOL FOR HPV DIAGNOSTICS

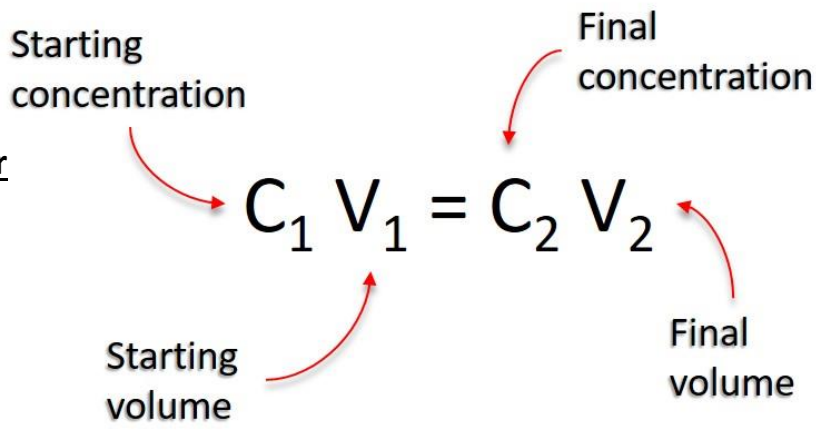
PART I Restriction reaction

Materials required:

DNA, restriction enzymes, thermomixer, pipettes, eppendorf tubes, loading dye

Reagent	Stock concentration	Final concentration/ amount	Final volume
DNA	20 ng/ul	100 ng/ul	
enzymes mixture	2 U/ul	0.5U/ul	
buffer	10x (concentrated)	1x (concentrated)	
water	up to 10 ul		

use general formula in order to calculate final volumes



Protocol:

Using a fresh tip for each reagent combine all in a fresh eppendorf tube in the given order:

1. water -> buffer -> enzyme -> DNA
2. Seal tube with the cap and label it.
3. Put the tube on a vortex to ensure proper mixing of reagents.
4. Spin down the tube in a small bench centrifuge.
5. Place samples into a thermomixer (set to 37 ºC) and incubate for 30 min.
6. After 30 min take the samples out of the thermomixer and add 5ul of the electrophoresis loading dye and mix gently by pipetting up and down.

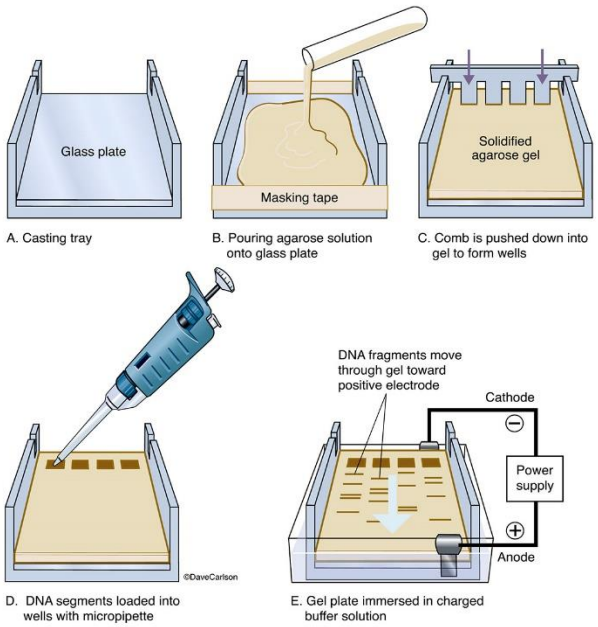
PART II Electrophoresis

Materials required:

agarose (powder), ethidium bromide, TBE buffer 1x, Erlenmeyer flask, analytical scale, microwave, gel casting plate, comb, electrophoresis tank, power supply

GEL PREPARATION

1. Calculate the amount of agarose necessary to make 50ml of 1% solution in TBE buffer.
2. Using the scale, weigh the necessary amount of agarose.
3. Measure 50ml of TBE buffer and pour it into an Erlenmeyer flask.
4. Put agarose into Erlenmeyer flask and swirl it.
5. Put the flask in a microwave and heat it for 5 min, making sure it does not overboil.
6. Cool the solution in RT (wait app. 10 min).
7. In the meantime prepare the casting plate and pour TBE buffer into the electrophoretic tank.
8. Once the agarose is cool add 1ul of provided ethidium bromide to Erlenmeyer flask and swirl it.
9. Pour the agarose into the casting plate.
10. Put an electrophoretic comb in the casting plate in order to create wells in the gel.
11. Leave the gel to cool down (you will know it's ready by milky color).



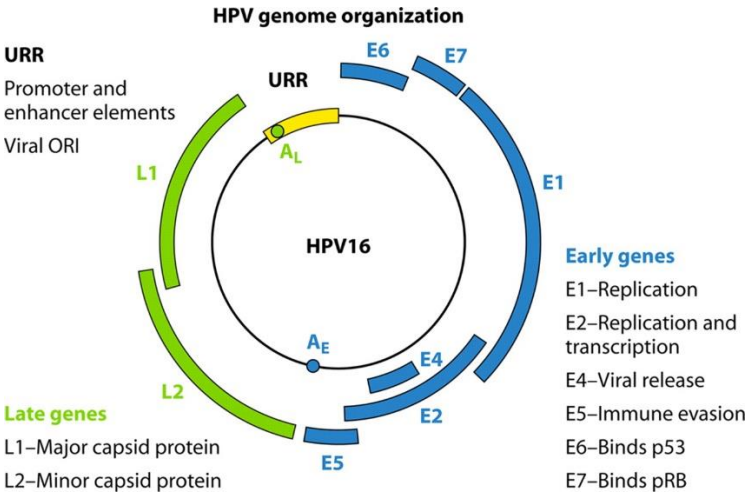
ELECTROPHORESIS

1. Once the gel is ready remove the comb and place the plate in the tank filled with TBE buffer.
2. Place your samples in the gel by carefully filling the wells.
3. Cover the tank with a lid and connect it to the power supply.
4. Run electrophoresis for app. 30 min applying 90V.
5. Analyze the gel using GBOX gel imaging system.

Human papillomavirus (HPV) and cervical cancer

Human papillomaviruses infect epithelial cells and generally induce the formation of benign hyperproliferative lesions (warts). Different types of HPV infection cause warts on different parts of the body. For example, some types of HPV infection cause plantar warts on the feet, while others cause warts that appear on the face or neck. Certain types of HPV infections contribute to the development of cervical cancers. HPV16 and HPV18 are widely accepted as the most carcinogenic genotypes. They are responsible for approximately 55–65% and 10–15% of all invasive cervical cancers (ICC) worldwide, respectively [Xu et al. 2018].

HPVs are oncogenic because they lack the DNA and RNA polymerases required for the viral life cycle and therefore must induce production of host cell replication proteins by driving the host cell into a proliferative state. To achieve this goal they use specific proteins which are encoded in the viral genome (picture below).



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The HPV oncoproteins E6 and E7 direct the progression of the cell cycle and play a major role in HPV-induced carcinogenesis by interfering with host cell regulatory proteins. The E7 protein inhibits the function of the retinoblastoma (Rb) family proteins, leading to cell cycle progression. In response to abnormal E7-driven proliferation, the host cell triggers apoptosis or senescence by activating the p53 tumor suppressor.

To defeat this antiproliferative host cell response, HPVs produce the E6 protein, which targets p53 for degradation [Stanley 2012; Hall et al. 2003]. E2 proteins are required for viral DNA replication but they also regulate viral gene expression. This way they can repress or activate transcription of the E6 and E7 oncogenes.

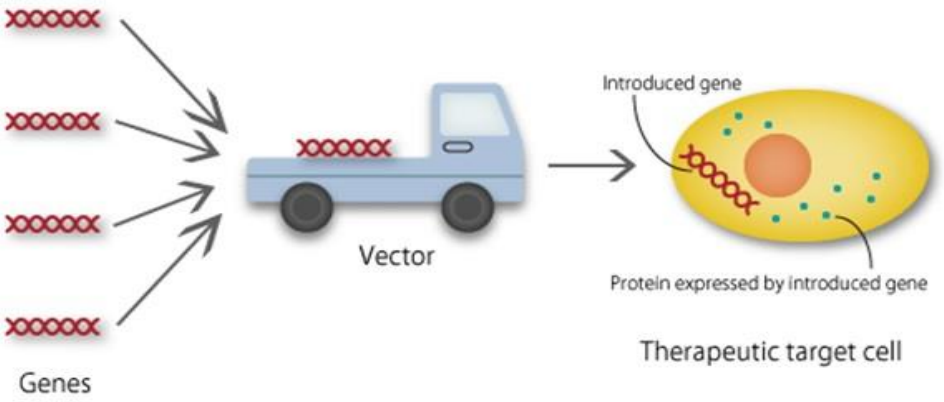
The E2 gene, or lack thereof, is thought to play a major role in the development of cervical cancer. Most cervical cancers contain chromosomally integrated copies of the HPV genome in which the viral E2 gene has been disrupted. Furthermore, mutations in the E2 gene increase the immortalization capacity of HPV 16.

Several laboratories have shown that the introduction of the HPV transcriptional regulator E2 into cells transformed with HPV induces either apoptosis or senescence of cancer cells [Desaintes et al. 1999].

Thus, the aim of the project is to perform gene therapy of human cervical cancer cells by introducting a copy of the human papillomavirus E2 gene into HeLa cells and analyse the consequences of E2 protein expression.

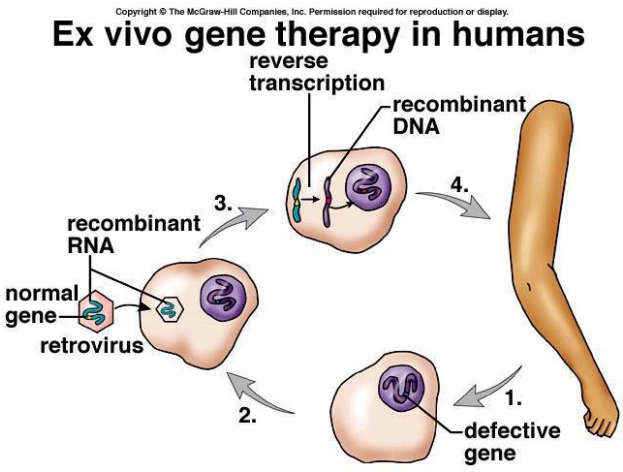
Gene Therapy

Gene therapy is an experimental technique for treating disease by altering the patient's defected genetic material. It derives its name from the idea that DNA can be used to supplement or alter mutated genes within an individual's cells.



Gene therapy involves the replacement (genetic defect complementation) or modification of a defective genetic variant to restore or enhance cellular function or to improve the reaction of non-genetic therapies. The other approaches involves: (i) inactivating, or “knocking out” a mutated gene that is functioning improperly; (ii) introducing a new gene into the body to help fight a disease.

There are two types of gene therapy:
(1) somatic cell therapy, in which human cells other than germ cells are genetically altered;
(2) germ line therapy, in which a therapeutic gene is integrated into the genome of human gametes or their precursors.



[http://highered.mheducation.com]

In order to obtain valuable clinical results, sufficient gene transfer is required. The retroviral and adenoviral vectors are the most frequently used in the clinic, but often plasmid DNA is also useful. Somatic cell gene therapy involves the transfer of therapeutic gene to a diseased somatic cell either within the body – in vivo or outside the body – ex vivo, with the help of a viral or non viral gene therapy vector. The therapeutic genes may be administered directly into target tissues or by intravenous or intramuscular injections.

Restriction Fragment Length Polymorphism

In 1968, Dr. Werner Arber at the University of Basel, Switzerland and Dr. Hamilton Smith at the Johns Hopkins University, Baltimore, discovered a group of enzymes in bacteria, which when added to any DNA will result in the breakage [hydrolysis] of the sugar-phosphate bond between certain specific nucleotide bases [recognition sites].

This causes the double strand of DNA to break along the recognition site and the DNA molecule becomes fractured into two pieces. These molecular scissors or “cutting” enzymes are restriction endonucleases. Restriction enzymes can be used to differentiate between DNA samples as well as detect changes in DNA sequence (mutations, SNPs).

Agarose gel electrophoresis

Agarose gel electrophoresis separates DNA fragments by size. DNA fragments are loaded into an agarose gel slab, which is placed into a chamber filled with a conductive buffer solution. A direct current is passed between wire electrodes at each end of the chamber. Since DNA fragments are negatively charged, they will be drawn toward the positive pole (anode) when placed in an electric field. The matrix of the agarose gel acts as a molecular sieve through which smaller DNA fragments can move more easily than larger ones. Therefore, the rate at which a DNA fragment migrates through the gel is inversely proportional to its size in base pairs. Over a period of time, smaller DNA fragments will travel farther than larger ones. Fragments of the same size stay together and migrate in single bands of DNA. These bands will be seen in the gel after the DNA is stained [source: BIORAD].