Polymerase Chain Reactions on Estrogen Receptor Knockout Mice

A Thesis

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by

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ABSTRACT

This research deals with estrogen receptor "knockout" (ERKO) mouse in which the gene for the estrogen receptor has been disrupted by gene targeting. These ERKO mice have no obvious external distinguishing features so it is necessary to determine the genotype of the offspring by polymerase chain reaction (PCR) amplification of gene specific sequences and visualization on ethidium bromide stained gel. I will be using a three primer technique in which all three genotypes (wildtype, heterozygote, and knockout) will result in a distinguishable pattern. This will allow for the possibility of using these combinations to determine the genotype of an unknown mouse in a single assay.

INTRODUCTION

In preparation of my CSP thesis/project, I have chosen to do research with polymerase chain reaction (PCR) test on estrogen receptor "knockout" (ERKO) mice. This research is being done in correlation with Kenneth S. Korach of the national Institute of Environmental Health Sciences. An estrogen receptor knockout mouse, in which the gene for the estrogen receptor has been disrupted by gene targeting, has been developed by scientist at the National Institute of Environmental Health Sciences. The ERKO mice can survive to adulthood with no distinguishing external features, therefore it is necessary to determine the genotype of the offspring by polymerase chain reaction (PCR) amplification of gene specific sequences. There are three possible genotypes in the mouse colony:

1. Wildtype: two estrogen receptor genes in which neither has been disrupted.
2. Heterozygote: one normal ER gene and one NEO gene which has been disrupted.
3. Knockout: two NEO genes in which both of the genes for the estrogen receptor has been disrupted by insertion of the neomycin receptor gene.

The PCR product of the wildtype gene is a 239 base pair fragment, while that of the disrupted gene contains 250 base pairs. Because it is not currently possible to amplify both fragments simultaneously, two separate PCR assays are now used in the genotyping of the mice. It is the goal of this project to use a three primer technique to detect the presence of both the wildtype estrogen receptor gene and the "knockout" estrogen receptor gene which has been disrupted by the insertion of a neomycin resistance gene.

IMPORTANCE

In the past, estrogen has always been looked at as just a hormone that causes female development. However, there is a lot more to estrogen. By activating specific receptor proteins, estrogen hormones can produce different actions within a variety of target sites in the body. Estrogen has been associated with the gonads, mammary glands, reproductive tracts, and skeletal tissues. Not only does estrogen play a vital role in female development, but embryonic and fetal development as well. As women get older and experience menopause, estrogen levels have been known to decrease. Osteoporosis and cardiovascular diseases have been linked to menopause. Therefore estrogen is believed to be linked with the functions of bone tissue and cardiovascular systems. Estrogen triggers responses which are tissue and organ-specific by binding to a nuclear receptor protein within target cells. Once the estrogen receptor knockout (ERKO) mouse is characterized, it should be useful in detecting different activities and responses of the estrogen gene. We are hoping this research will be significant in the scientific development of the future. By studying such animals as mice and the effect the ERKO has on them, we can hopefully extrapolate this to humans. (Korach, 24-27)

EXPERIMENTAL

METHODS

The first thing we are concerned with is that we must determine the genotype of each animal by two major methods: polymerase chain reaction (PCR) and electrophoresis.

POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is considered one of the most significant DNA technologies to be developed. PCR uses enzymes and raw materials to copy part of a genome and amplify tiny amounts of DNA. A few micrograms of DNA can be amplified millions or billions of times in two to three hours with this technique. This allows researchers to do DNA analyses form any organisms, even when very little DNA is available. Polymerase chain reaction uses a method of DNA replication similar to that used by every living organisms in "natural" DNA replication in that the polymerase enzyme helps to construct new DNA. The polymerase chain reaction takes place in small tubes, with a

mixture of DNA template (sample), DNA polymerase enzyme, and other substances.

The efficiency of PCR depends on several reaction parameters including: polymerase, magnesium concentration, primers, amount of sample, number of cycles, starting temperatures, and pH. Magnesium concentration is one of the most important parameters in PCR. An overabundant amount of magnesium may result in a variety of unwanted products and too little magnesium may result in small or no PCR product. Usually the magnesium concentration that will produce the best yield of PCR product is determined by performing the PCR reaction over a series of magnesium concentrations in 0.5mM increments.

Primers are also an important factor in the reaction. Primer pair 2382 and 2383 (3 and 4) when used with a reaction buffer of pH 9.5 and a Mg concentration of 7.5mM, this primer pair results in the amplification of a 239 base pair fragment characteristic of the wildtype gene. Primer pair 2086 and 2618 (5 and 6) when used with a reaction buffer of pH 9.5 and a Mg concentration of 10mM, this primer pair results in the amplification of a 250 base pair fragment characteristic of the disrupted gene.

Another major parameter in PCR is the thermocycle. In the

thermocycle the PCR mixture is exposed to three different temperatures, constituting one PCR cycle. Usually 20-40 cycles compromise one PCR amplification experiment. Each cycle results in amplification of products from the previous cycle. The first phase of the PCR cycle is called the denaturing step in which double stranded DNA is separated into two single strands by heating the PCR mixture to approximately 94 degrees celsius. The second phase is called the annealing phase. The temperature is reduced to approximately 50 degrees celsius, allowing DNA "primers" to anneal to specific sites on the large single strands of DNA. Once these primers have annealed, the solution is brought to approximately 72 degrees celsius, and the final phase or extension phase begins. During extension, polymerase enzyme causes loose deoxynucleotide triphosphates (dNTPS) to base pair with single stranded DNA beginning at the primer position, creating two identical double stranded DNA molecules from one. The number of DNA molecules is doubled with each cycle. (Cano, 478-481)

ELECTROPHORESIS

Electrophoresis is a technique used to separate and identify fragments of DNA. The DNA sample is loaded into its own individual well of the agarose gel and hooked to a voltage supplier with a negative and positive charged end. The electric current is applied and forces the sample down the gel based on its charge at a specific time. The migration rate of a sample depends on several factors.

Agarose concentration in the gel is one of these factors. The more agarose contained in the gel means the more dense or stiff the gel will be. The more dense the gel is the slower large fragments of DNA will move. Therefore, the ideal method of using the gel would be to use small fragments of DNA in gels with high concentrations of agarose and large fragments of DNA in gels with low concentrations of agarose.

The DNA fragments have an optimum voltage range at which they will separate accurately. Therefore, the amount of current applied and the period of time the current is applied will affect the migration rate of the DNA fragments. At low voltages, the distance traveled is proportional to the amount of voltage applied. At high voltages, the mobility of the DNA decreases. (Maniatis, 17-214)

PROCEDURE

OVERALL PROCEDURE:

1. Extraction of DNA from the mice

   A piece of the tail of the mouse is snipped, subjected to enzymatic degradation and the DNA extracted by chloroformphenol.

   NOTE: This step has already been done by technicians at the NIEHS.

2. Preparation of reaction mixtures
3. Setting up and running the PCR
4. Separation of PCR products by agarose gel electrophoresis

REAGENTS:

1. PCR WATER
2. Buffer J
3. 10X deoxynucleotide mixture (dNTPs)
4. TAQ Polymerase
5. DNA samples from mice of "known" genotypes
6. Primer 2382 (3), 2383 (4), 2086 (5)

Setting up the reaction mixtures:

Preparation of reaction mixtures involves preparing a "lower buffer" and an "upper buffer". The total volume of the lower buffer will be 13 microliters per sample and the total volume of the upper buffer will be 10 microliters per sample. With the addition of 2 microliters of sample, the total reaction volume is 25 microliters.

Each of the lower and upper reaction mixtures will be prepared in a "master mix" to minimize pipetting error. You should calculate how many reactions you are to run (in this case it will be 6), and then add 2 so you will be sure to have enough mixture. Then multiply that number by 13 for the lower buffer and 10 for the upper buffer. The result will be the total number of microliters of each buffer that you must prepare.

Preparation of lower buffer master mix:

Reagent	amount per rxn. (microliters)	total amount (10 rxns.) (microliters)
PCR Water	6.0	48
Buffer J	3.0	24
Primer 2282 (3)	0.5	4
Primer 2383 (4)	1.0*	8
Primer 2086 (5)	0.5	4
10X dNTPs	2.0	16
Total Volume	13.0	104

*Add twice as much primer 2383 because it will work in two ways (a 239 base pair PCR product will be formed between primers 3 and 4: indicative of the ER gene, and a 250 base pair product will be formed between 4 and 5: indicative of the NEO gene.

Preparation of upper buffer master mix:

Reagent	amount per rxn. (microliters)	total amount(10 rxns.) (microliters)
PCR Water	7.75	62
Buffer J	2.0	16
TAQ Polymerase**	.25	2
Total volume	10.0	80

**Do not add the TAQ until the tubes have been prepared for insertion into the thermocycler.

After preparation of the upper and lower buffers:

Turn on the thermocycler so that it can be warming to a temperature of 80 degrees celsius. Push "Run Knockout".

Assay is to be run on six samples:

Four known heterozygotes: Sample No. 34-30, 44-01, 45-13,47-18

One known knockout: Sample No. 39-24

One known wildtype: Sample No. 34-15

Label six PCR tubes with sample numbers above.
Into each tube pipette 13 uls of the lower buffer mix.
Pipette 2 uls of sample into the appropriate tubes.
Add the TAQ polymerase to the upper buffer master mix
(NOTE Above **)

Pipette 10 uls of the upper buffer mix into the firs tube and IMMEDIATELY add 50 uls of corn or mineral oil and place into the thermocycler which is ALREADY WARMED TO A TEMPERATURE OF 80 DEGREES CENTIGRADE. Repeat the procedure for the next tube and continue until all of the tubes have been placed in the thermocycler.

The thermocycler is programmed to run through 40 of the following cycles:

95 degrees for one minute (DNA denaturation step)
55 degrees for two minutes (Primer annealing step)
72 degrees for thirty seconds (DNA synthesis step)

After 40 cycles the thermocycler will go to 4 degrees centigrade and hold. The samples may be left at that temperature indefinitely.

The entire thermocycler run will take about two and one half hours.

Preparation of 2.8% metaphor gel:

The gel should be made up the same day as the PCR run and left in the refrigerator overnight.

The gel needs to be about 6.25 mm thick. Therefore if the gel tray is 150X150mm need to make 140mls of gel.

Thus to make a 2.8% metaphor gel you will need to take 3.92 grams of metaphor and add to 140mls of 1X TBE

FORMULA FOR 10X TBE (one liter):

108 grams of Tris Base
55 grams of Boric acid
40 mls of .5M EDTA (pH 8.0)

After you have prepared the gel mixture, boil or microwave it to dissolve the metaphor. Add 7 microliters of ethidium bromide solution.

CAUTION: ETHIDIUM BROMIDE IS A SUSPECTED CARCINOGEN, ALWAYS WEAR GLOVES WHEN WORKING WITH IT OR WHEN WORKING WITH GEL THAT CONTAINS ETHIDIUM BROMIDE.

To make ethidium bromide solution, dissolve 100mgs of ethidium bromide in 10mls of water.

After the ethidium bromide has been added to the gel, pour the still liquid gel into a gel tray, place a well comb and wait until it solidifies. You can store this in the coldroom over night if you place it in 1X TBE to prevent it from drying out.

Loading the samples on the gel:

Add 3 microliters of tracking dye (6X TCD) to each sample. Put the dye ont eh side of the tube above the sample and gently tap the tube so that it falls into the sample. Do not put the dye into the sample!

Load 20 microliters of the sample (with tracking dye) into appropriate wells on the gel. Place 20 microliters of PCR markers into an empty well near the middle of the gel.

Samples should be loaded as follows:

Lane	Sample
1	34-15 (wildtype)
2	34-30 (heterozygote)
3	44-01 (heterozygote)
4	PCR markers
5	45-13 (heterozygote)
6	47-18 (heterozygote)
7	39-24 (knockout)

Subject the gel to a current of 100 volts for two hours. Set both wattage and current on 100 then turn voltage until it reads 100.

If all works well in two hours the PCR products of 239 and 250 nucleotides should be resolved. Turn off the power and remove gel. Visualize the PCR products with the UV transluminator: CAUTION ALWAYS USE PROTECTIVE EYEWEAR WHEN THE TRANSLUMINATOR IS IN USE. DO NOT LOOK AT THE TRANSLUMINATOR WITH YOUR UNPROTECTED EYES!!!

If the PCR products have not been resolved sufficiently place the gel back into the electrophoresis unit and continue the electrophoresis.

After the final electrophoresis, make a photograph of the gel for your records. Be sure to write on the back of the photograph: the date and information about which sample was add to each well.

DATA

Experiment #1
11/15/95

Objective: To use the polymerase chain reaction and electrophoresis techniques to detect the presence of both the ER and NEO gene using primers 3,4,and 5 in conjunction with my six samples.

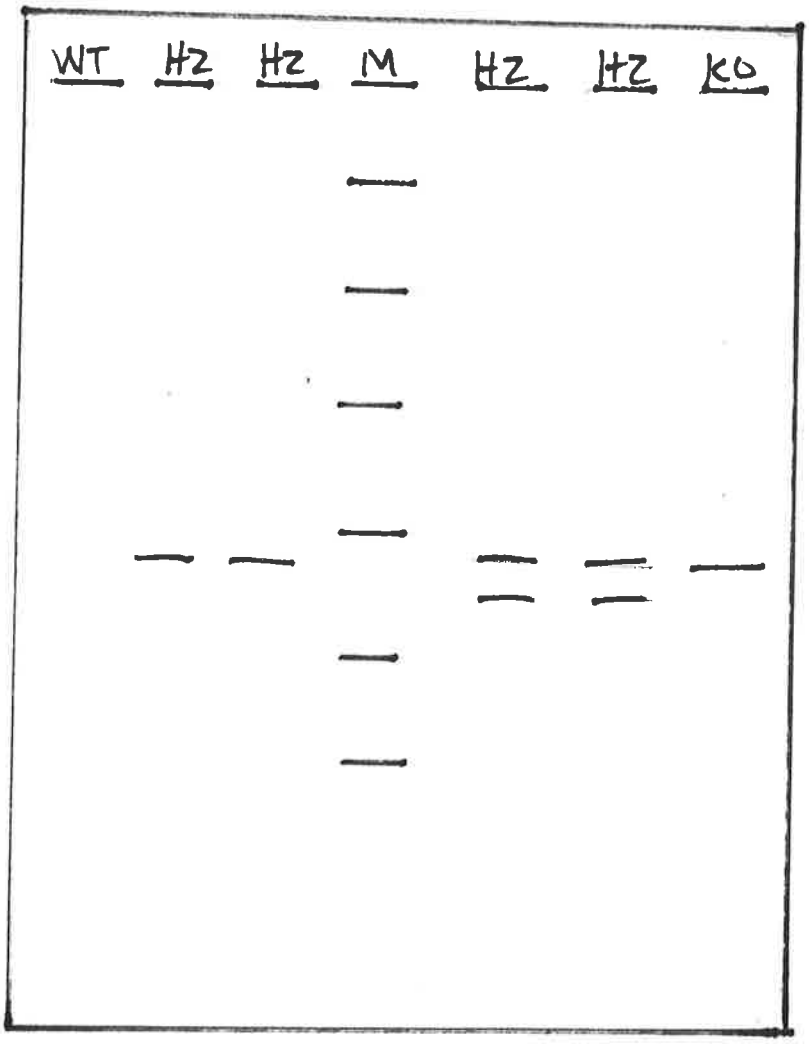
Procedures: Follow outline of procedures listed on previous pages.

Results: Photograph of results on following page.

Lane 1 (wildtype): no band reading developed.
Lane 2 (heterozygote): developed a 250 base pair.
Lane 3 (heterozygote): developed a 250 base pair.
Lane 4 (PCR marker): developed line of marker bands.
Lane 5 (heterozygote): developed both a 239 & 250 base pair.
Lane 6 (heterozygote): developed both a 239 & 250 base pair.
Lane 7 (knockout): developed a 250 base pair.

Conclusion: My first three lanes proved unsuccessful for their expected products. The last three lanes developed the base pair reading that were expected. This experiment worked very well for my first try.

Experiment #1
11/15/95



Experiment #2
11/22/95

Objective: To use the polymerase chain reaction and electrophoresis techniques to detect the presence of both the ER and NEO gene using primers 3,4,and 5 in conjunction with my six samples.

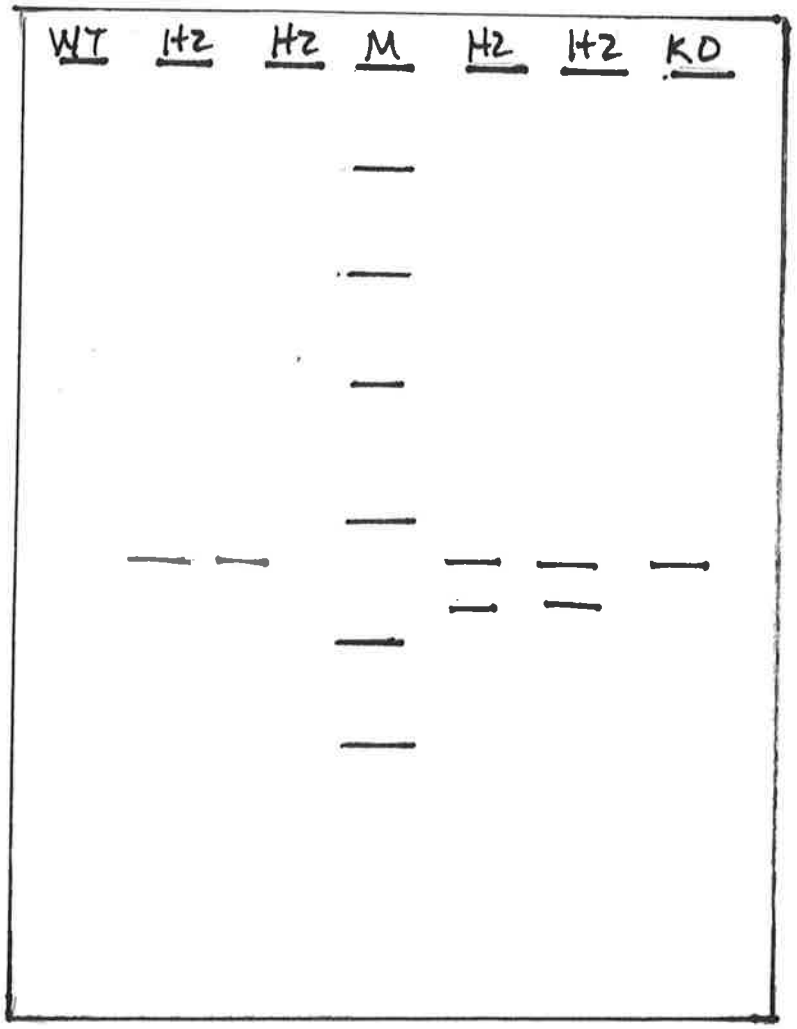
Procedures: Follow the outline of procedures stated on the previous pages.

Results: Photograph of results on following page.

Lane 1 (wildtype): no band reading developed.
Lane 2 (heterozygote): developed a 250 base pair.
Lane 3 (heterozygote): developed a 250 base pair.
Lane 4 (PCR marker): developed marker bands.
Lane 5 (heterozygote): developed both a 239 & 250 base pair.
Lane 6 (heterozygote): developed both a 239 & 250 base pair.
Lane 7 (knockout): developed a 250 base pair.

Conclusion: My first three lanes proved unsuccessful for their expected products. The last three lanes showed the base pair products that were expected.

Experiment #2
11/22/95



Experiment #3
1/29/96

Objective: To use the polymerase chain reaction and electrophoresis techniques to detect the presence of both the ER and NEO gene using primers 3,4,and 5 in conjunction with my six samples.

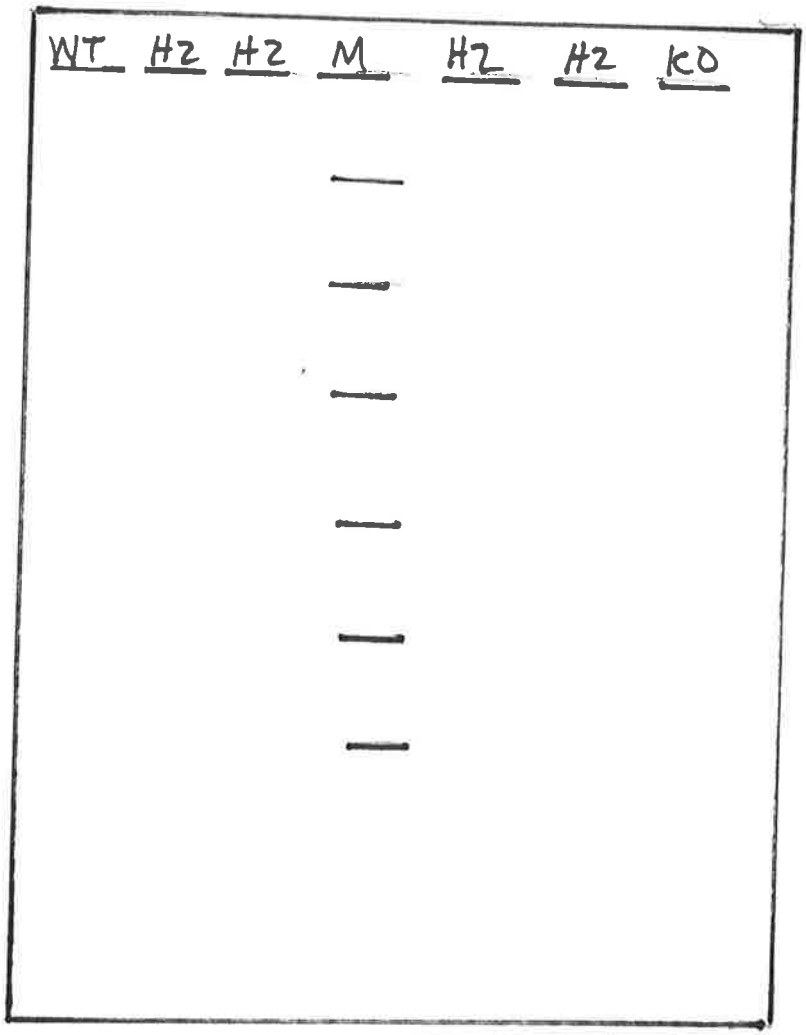
Procedures: Follow outline of procedures stated on previous pages.

Results: Photograph of results on the following page.

Lane 1 (wildtype): no band reading developed.
Lane 2 (heterozygote): no band reading developed.
Lane 3 (heterozygote): no band reading developed.
Lane 4 (PCR marker): no band reading developed.
Lane 5 (heterozygote): no band reading developed.
Lane 6 (heterozygote): no band reading developed.
Lane 7 (knockout): no band reading developed.

Conclusion: This procedure was not successful at all. I got no results at all. I have concluded that I must have made some error while mixing the reagents. I think this error occurred when adding the ethidium bromide solution to my mixture.

Experiment #3
1/29/96



Experiment #4
2/13/96

Objective: To use the polymerase chain reaction and electrophoresis techniques to detect the presence of both the ER and NEO gene using primers 3,4, and 5 in conjunction with my six samples.

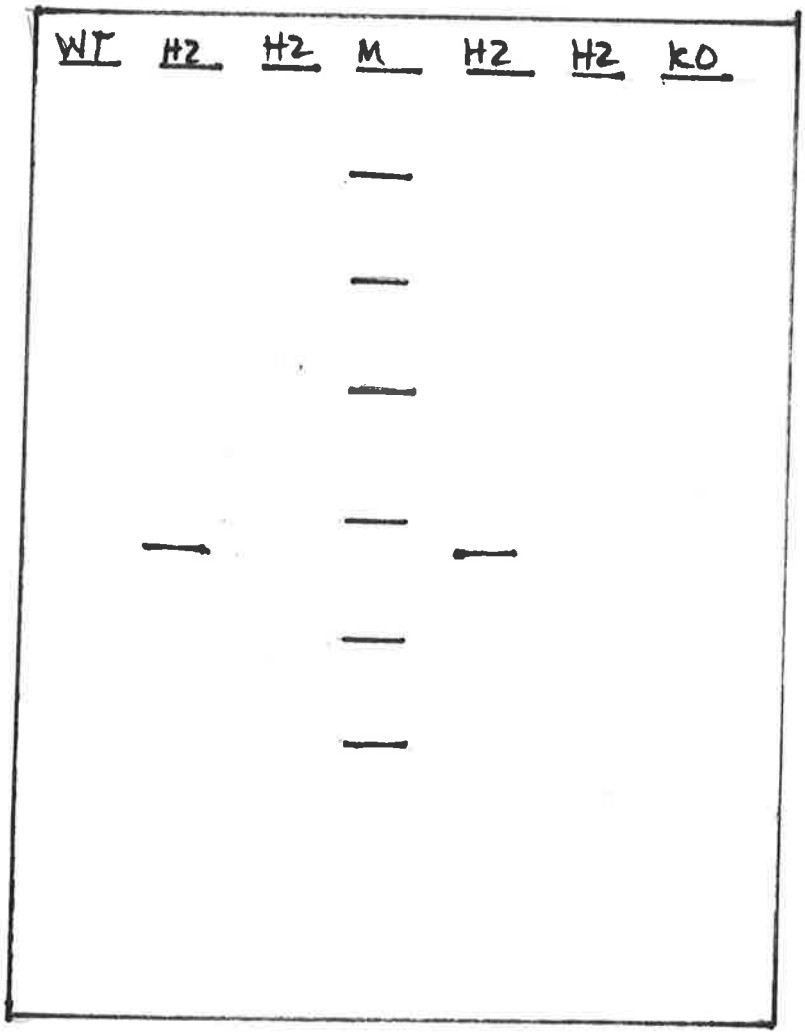
Procedures: Follow outline of procedures stated on preceding pages.

Results: Photograph of results on following page.

Lane 1 (wildtype): no band reading developed.
Lane 2 (heterozygote): developed a 250 base pair.
Lane 3 (heterozygote): no band reading developed.
Lane 4 (PCR marker): no band readings developed.
Lane 5 (heterozygote): developed a 250 base pair.
Lane 6 (heterozygote): no band reading developed.
Lane 7 (knockout): no band reading developed.

Conclusion: The base pairs in this gel did not separate out that well. From my photograph it was very hard to read the results. The PCR marker did not show up at all either so I guessed at the two bands that did show up.

Experiment #4
2/13/96



Experiment #5
3/18/96

Objective: To use the polymerase chain reaction and electrophoresis techniques to detect the presence of both the ER and NEO gene using primers 3,4, and 5 in conjunction with my six samples.

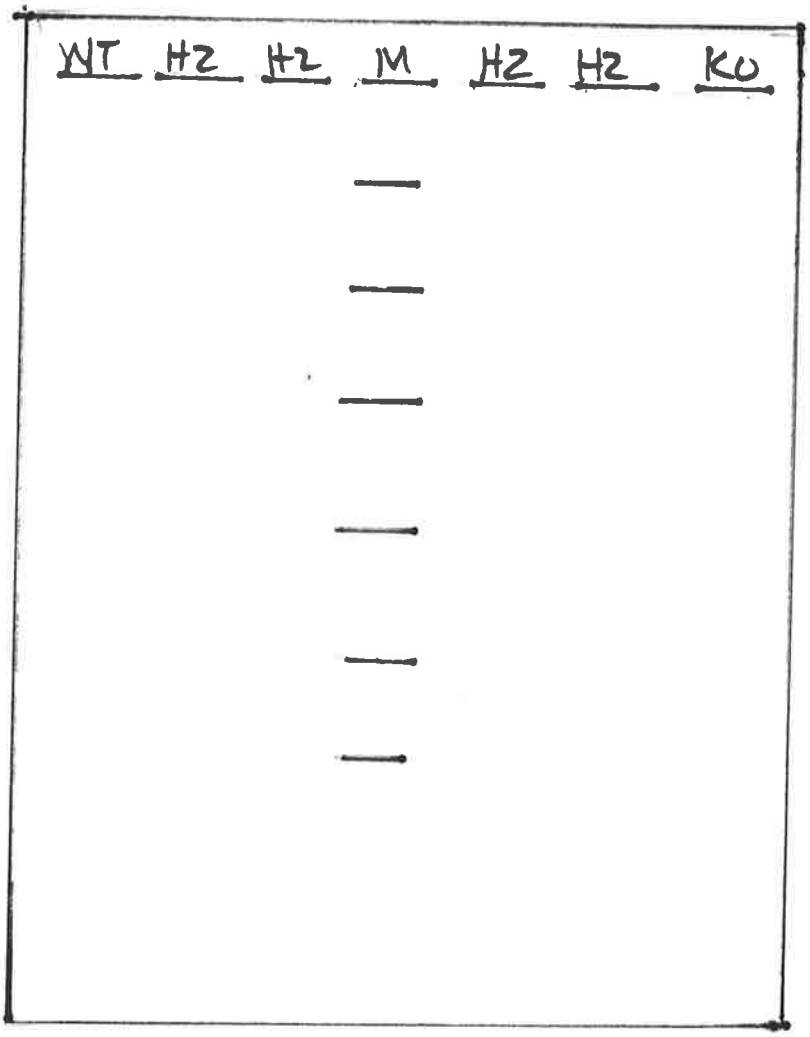
Procedures: Follow outline of procedures stated on preceding pages.

Results: Photograph of results on following page.

Lane 1 (wildtype): no band reading developed.
Lane 2 (heterozygote): no band reading developed.
Lane 3 (heterozygote): no band reading developed.
Lane 4 (PCR marker): no band readings developed.
Lane 5 (heterozygote): no band reading developed.
Lane 6 (heterozygote): no band reading developed.
Lane 7 (knockout): no band reading developed.

Conclusion: This procedure was not successful at all either. Once again I ended up with no products. I have concluded that once again I have made an experimental error.

Experiment #5
3/18/96



Experiment #6
3/28/96

Objective: To use the polymerase chain reaction and electrophoresis techniques to detect the presence of both the ER and NEO gene using primers 3,4, and 5 in conjunction with my six samples.

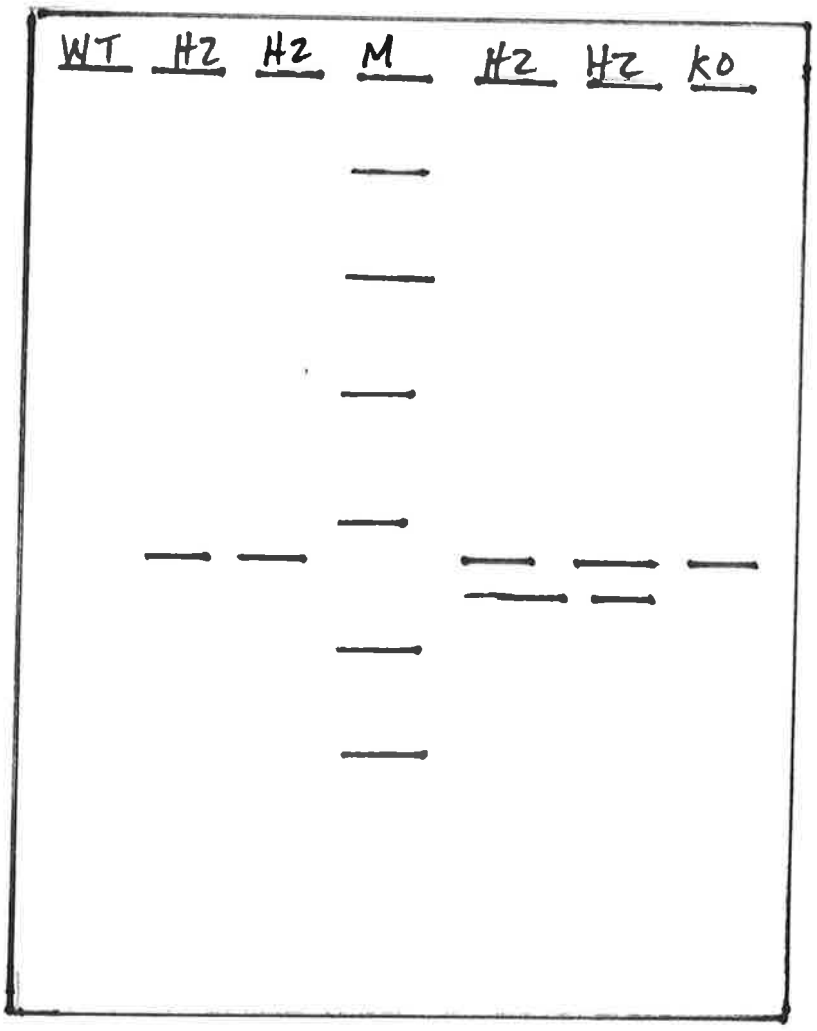
Procedures: Follow outline of procedures stated on preceding pages.

Results: Photograph of results on the following page.

Lane 1 (wildtype): no band reading developed.
Lane 2 (heterozygote): developed a 250 base pair.
Lane 3 (heterozygote); developed a 250 base pair.
Lane 4 (PCR marker): developed a line of marker band.
Lane 5 (heterozygote): developed both a 239 & 250 base pair.
Lane 6 (heterozygote): developed both a 239 & 250 base pair.
Lane 7 (knockout): developed a 250 base pair.

Conclusion: My last experiment proved to be an exact repeat of my first one. It was half successful in the fact that the first three lanes did not produce the correct products but the last three lanes did develop the expected base pair products.

Experiment #6
3/28/96



Overall Conclusion

In conclusion, my project was an overall success. My experiments proved that there is a way to test for both the ER and NEO gene in a single assay. Although three of my samples did not work, two of the samples showed both base pairs in all of the successful experiments. The three samples that did not work (the wildtype, and the first two heterozygotes), will be rechecked to insure that they are correctly marked. I hope my research will be a building block to be used in future study of this project.

ACKNOWLEDGEMENTS

I would like to take this time to thank all the people who have helped me in the completion of this thesis. First, I would like to thank Dr. Jenkins for allowing me to be apart of the Chancellor's Scholars Program. I would like to thank Dr. Maxwell, my faculty advisor, for being patient with me throughout my research, in its success and its failures. Last but not least, I would like to thank Dr. Williams, Dr. Brown, and the CSP council members for their assistance with the completion of all the requirements for this program.

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