The Effects of Phytoestrogens on Breast Epithelial Cells

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Degree of Bachelor of Science Submitted by:

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Abstract

Phytoestrogens are found in over the counter hormone replacement therapies and may react with the estrogen receptor in a manner similar to estrogen. A strain of MCF7 breast epithelial cells was cultured and assessed using an MTT cell proliferation assay. Assays were performed with combinations of IGF and estrogen to determine estrogen responsiveness. Although the strain of cells was not estrogen responsive, further testing on a different strain could determine how the phytoestrogens affect proliferation rates in comparison to estrogen.

Acknowledgments

We would like to thank several people for making this project what it is. First we would like to thank our advisors, Mike Buckholt and Jill Rulfs for their guidance and expertise in working with cell culture projects. Their knowledge of troubleshooting problems in the experiment was incredibly helpful and allowed us to try almost everything before the project was over. Second, we would like to thank Abbie White for her help in ordering supplies for our project. Without her help, we would not have been able complete this project. Third, we would like to thank Casey Raasumma and Jess Caron for the work they have done previously on this topic. Their papers were used as our starting point, and without prior knowledge of what was already done, we would have found ourselves repeating the same failed experiments. Fourth, we would like to thank JoAnn Whitefleet-Smith for allowing us to use the cell culture room and drawers, and to the cell culture TA's, Jeff Swana and PingPing Ren, for allowing us to use the cell culture hood during class if we needed to. Without the cooperation and helpfulness of all of these people, our project would not have been possible. Again, thank you.

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Introduction

Over the past several years, the idea that over the counter phytoestrogens could be used as hormone replacement therapy (HRT) remedies was brought to the attention of scientists. The benefits of phytoestrogen treatment versus traditional replacement therapy could prove to be quite beneficial to many women who suffer from the negative side effects associated with menopause and hormone replacement therapy.

The goal of this project was to study the effects of phytoestrogens on a breast epithelial cell line called MCF-7's. First, in order to test the effects of phytoestrogens, the cells had to be validated as being estrogen responsive. In this project, we obtained frozen stocks of ATCC MCF-7's from a previous MQP (Raasumaa, 2008), frozen at passage 7. These stocks were thawed and cultured in order to gain enough cells to plate and test.

Once plated, the cells were tested for proliferation using Promega's Cell Titer 96 Aqueous Solution Cell Proliferation Assay Kit. This assay was used to determine the number of viable cells through optical density. The cell titer kit uses an MTS tetrazolium compound, also known as Owen's Reagent, which is bioreduced using NADPH and NADH from metabolically active cells into a colored formazan dye. The formazan product is soluble in the cell culture media, turning the media in the plate purple. The concentration of this dye is directly proportional to the number of livings cells in the media. The darker the dye, the higher the optical density reading, and presumably, the higher the proliferation rate of the cells.

In order to learn the growth kinetics or growth parameters of the cells, the first assays performed were controls for the experimental design. This data lead to the promising hypothesis that the cells were estrogen responsive due to their proliferation in Phenol Red DMEM. Through further testing however, it became clear that the cells were not estrogen responsive. After stimulating the cells with varying levels of estrogen, IGF, and varying the cell concentrations, absorbencies did not change as expected. Thus the MCF-7 cells used were not responsive, and therefore not a useful model for testing phytoestrogen effects.

The next step, for further years to come, will be to choose a different cell line and repeat the tests performed in this project.

Background

Phytoestrogens are thought to have an effect on the proliferation of breast epithelial cells. This effect is very similar to hormone replacement therapies which are currently used in medicine today to treat menopause symptoms. These symptoms include vasomotor symptoms such as night sweats and hot flashes, decreased bone density which increases risk of bone damage, change in mammographic breast density, and increased risk of cardiovascular disease and stroke. The specific therapy treatments vary and drugs commonly include phytoestrogens, natural estrogen supplements, and dietary supplements, as well as simple placebos. These are all intended to be used by the body similarly to estrogen to prevent menopause symptoms.

Specific therapy treatments vary. The drugs which are used include phytoestrogens, natural estrogen supplements, and dietary supplements, as well as simple placebos. The specific phytoestrogens include promensil and rimostil. Natural estrogen supplements include simple estrogen and simple estrogen with progestin. However, these are more effective when given with steroidal supplements. Dietary supplements are given in the form of red-clover-derived isoflavin which contain genistein, among other chemicals.

Estrogen is the primary female reproductive hormone important for all aspects of female development. This steroid hormone is derived from cholesterol and transported throughout the bloodstream to target areas of the body. There are three types of estrogen: estradiol, estrone, and estriol. Estradiol is the most prominent form in the body. Estrogens have many effects on the female body; they are present in numerous areas of the body such as the brain, heart, liver, breasts, uterus, ovaries, vagina, and bones (Britannica, 2005). The hormone has different effects in each area, from preparing organs for maturation, preservation of conditions needed for child bearing, maintenance of bone density, and regulation of cholesterol and body temperature (Hospital, 2008). Figure 1 shows a summary of the areas estrogen affects the female body.

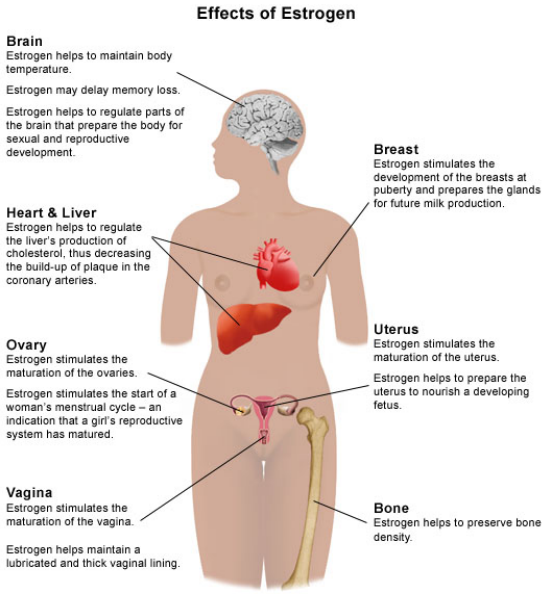


Figure 1: Effects of Estrogen on the Female Body (Hospital, 2008)

Since estrogen is such an important hormone, any increases or decreases in its concentration may have detrimental effects. One such effect is during menopause when the decrease in estrogen causes many unpleasant side effects such as headaches, hot-flashes, nausea, and vaginal discomfort (Medicine, 2008). In order to alleviate these sometimes debilatating symptoms, women turn to hormone replacement therapy. There are several types of hormone replacement therapies that a women can take. These can be estrogen or estrogen-progestin regimens, but women are cautioned to take the lowest dose needed to alleviate their symptoms (Medicine, 2008).

This therapy has risks, however, which is why the effects of phytoestrogens are currently being investigated. Some of the risks of hormone replacement therapy include increased risk of heart disease, stroke, breast cancer, and blood clots (Medicine, 2008). Why then would a woman want hormone replacement therapy? The potential risks associated with the treatment, although of concern to the entire population, are quite small per individual woman (Medicine, 2008). One of the risks of HRT which is relevant to this study, is the increased risk of breast cancer. In an article published in the Journal of the American Medical Association, Shairer et al indicated an increase in breast cancer risk during each year HRT was used (Schairer, 2000). The article also showed a larger risk of breast cancer for estrogen-progestrone than for estrogren HRT alone. Although studies are showing that there are risks to using HRT, there are also benefits to using the hormones and women must consider all the benefits and risks when considering the use of HRT.

Because of the many risks of hormone replacement therapy, there is cause to search for other treatments with some of the same benefits of HRT but without the inherent risks. One

such alternative may come in the form of phyoestrogens. These plant-derived substances are functionally and structurally similar to estradiol and thus may be used as an alternative for estradiol-based HRT treatments (Duffy, 2007). Present ly,the data on phytoestrogens is lacking and more research is needed to determine the true effects of these plant-based compounds on breast cancer risk reduction and protective measures that some have claimed these substances have (Duffy, 2007). Many women may turn to phytoestrogen products such as the over-the-counter drug Promencil® because they are considered to be natural. It is still unknown, however, whether natural methods are safer and more beneficial than closely regulated treatments. Such information can only be determined with more research.

Duffy et al (2007)studied topics related to phytoestrogens' effects on the human body. There is very little estrogen activity per mole of phytoestrogens compared to estrogen alone. This is measured in several different ways including their ability to modulate transcription of target genes, breast cancer cell growth, and affinity for particular ERs in the body. However, the concentration of naturally occuring phytoestrogens in the female body is much higher than that of estradiol (Duffy, 2007). This higher concentration has lead researchers to believe that 17β-estradiol and phytoestrogens may work in the same ways in the female body despite the fact that there is less activity with phytoestrogens than estrogen. There are reservations about phytoestrogens, however because questions about dosing, estrogenic activity, and any beneficial effects of phytoestrogens versus HRT must be further researched and analyzed (Duffy, 2007). More research on the topic aims to clear these issues up.

In order to understand how 17β-estradiol and phytoestrogens (particularly the estrogen, genistein) can affect menopausal symptoms, it is important to note the biochemistry of the two compounds. The compounds are structurally similar as noted in Figure 2.

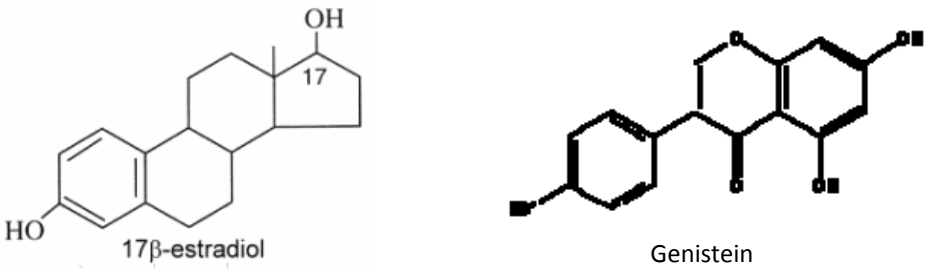


Figure 2: Structure of 17B-estradiol (left) and Genistein (right)

Because the compounds are similar, their mechanisms of action are also quite similar. Estrogens and phytoestrogens bind to alpha and beta estrogen receptors. The binding to the estrogen receptors then activates the genes responsible for the estrogenic effects. These effects include breast cell growth which may lead to disease.. In a paper by Delaunay et

al(2000), it was noted that both receptors are structurally similar in their binding domains. However, the beta receptor has a higher affinity for some phytoestrogens such as genistein (Delaunay, 2000). Because of this, it is important to study the effects of genistein on estrogen receptor positive cell lines, such as the MCF-7 line which is reported to both express the estrogen receptor beta and respond to estrogen with an increase in cell proliferation..

The MCF-7 cell line, which are adherent breast epithelial cells, was originally obtained from a 69 year old caucasian woman's breast tumor. The ATCC's cytogenic analysis yeilds a karyotpye mode of 82 and a range of 66-87 (ATCC, 2008). The line has retained several characteristics of breast epithelial cells that have undergone differentiation, including the ability to transport estradiol through estrogen receptors in the cytoplasm. Numerous strains have been developed since the cell line's isolation in 1970. These strains have been known to posess differing levels of estrogen responsiveness, although the cause of these differences is still unknown.

Research conducted on three different strains of MCF-7 cells showed that estrogen responsiveness at an estrogen concentration of 1 nM can increase proliferation rates, and this affect is amplified by the addition of Insulin-like Growth Factor 1 (IGF-1)(Hamelers et al, 2003). In previous studies, the estrogen responsiveness of MCF-7 cells from the ATCC was found to be less than anticipated as the addition of varying amounts of estrogen to the cell cultures resulted in no differences in proliferation rates.(Hamelers et al, 2003) It is thought that perhaps passage number could play a role in varying estrogen responsivenesses (Raasumaa, 2008 and Caron, 2007 and Hamelers et al, 2003). Research suggests that prolonged culturing in the absence of estrogen may select for a subpopulation of cells that are able to proliferate rapidly without the estrogen that would be present in an *in vivo* setting (ref). Cells grown in the absence of estrogen for extended amounts of time have lower levels of estrogen-stimulated proteins such as the progesterone receptor. These cells do not increase their proliferation rates with the addition of estrogen, although they are anti-estrogen suppresable (Katzenellenbogen, 1987). Studies such as Hamelers et al (2003) have shown that there is a correlation between highly passaged cells grown in the absense of estrogen and a lack of estrogen responsiveness.

Receptors for IGF-1, Insulin-like Growth Factor (IGF), have been found in estrogen-positive breast tumors such as the one that the MCF-7 cells were derived from. Pratt and Pollack (1993) showed that IGF-1 significantly increased the proliferation rates when used in conjuction with estradiol, and that this effect was due to the increase in insulin-like growth factor binding proteins such as BP-4 in the presence of estrogen, which show a positive correllation with proliferation rates . These IGF's are only some of the many different types of growth factors secreted by MCF-7 cells. There are also a wide variety of hormone receptors found on MCF-7 cells that include, but are not limited to, both estrogen and progesterone. Hamlers et al (2006) also suggested that IGF may be influential in the cell proliferation that is induced by estrogen. They further suggest that ifferences in autocrine signaling through the IGF

receptors amay be responsible for the significant differences in estrogen responsiveness seen among different strains of MCF-7 cells.

Recent studies of the MCF-7 cell line have shown that this previously consistent model system for studying breast cancer is no longer consistent in estrogen responsive proliferation. It is concluded that these strains must first be recharacterized before consideration for use in a breast cancer study. The data shows that the ATCC MCF-7 cells after at least passage 7 are no longer estrogen responsive. The data is also showing unresponsiveness even with the exogenous addition of IGF I or IGF II.

Materials and Methods

Media:

Several different media were used throughout the project. Primarily, cells were kept in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS). Immediately after thawing fresh cells, 20% FBS DMEM was used to stimulate growth. At times when there was a significant decrease in the number of cells needed and to reduce frequent passaging, 5% FBS DMEM was used. For freezing down stocks of cells, a freeze media containing 10% DMSO, 10% FBS DMEM was created.

Assays were performed in Phenol red free DMEM with 10% dextran-coated charcoal stripped FBS (PHRED). The phenol red was eliminated so as to reduce its interaction with the estrogen receptors of the MCF-7 cells, which could have resulted in a false positive reading.

Cells:

MCF-7 cells originally from the ATCC were thawed from passage 7 of a previous MQP at a concentration of $1x10^6$ cells/mL. The cells were originally plated in a T25 and then expanded up to a T75 after the first passaging. The media was changed 2-3 times a week by aspirating off the old media and replacing it with 12 mL of 10% FBS DMEM. At each media change, cells were checked for confluency and overall appearance.

Cells were passaged upon reaching approximately 95% confluence. The media was first aspirated off, and then the flask was rinsed with 5mL of 1x PBS. 1.5mL of 1X trypsin was then added to the flask and allowed to sit for 3-5 minutes, or until the cell layers had visibly detached from the flask. If cells had not detached by 5 minutes, a rubber policeman was used to manually scrape cells from the flask. 5mL of 10% FBS DMEM was then added to inactivate the trypsin and suspend the cells. This volume was collected in a 15 mL tube and spun down for 5 minutes. The new flask was plated at a concentration of 1:10 of the original, and remaining cells were either used for assays, frozen down to keep as stocks, or discarded.

In order to freeze cells as stocks for future use, cells were counted after the trypsinizing process. $1x10^6$ cells were then frozen in 1mL of freeze media, kept in the -80^oC freezer for 1 day, and then moved to the liquid nitrogen storage unit. Cell stocks were frozen every 10-15 passages.

Reagents:

IGF was dissolved in a solution of 10mM Glacial Acetic Acid with 0.1% BSA. After going into solution, it was diluted to a concentration of $5x10^4$ng/mL. The final concentration in the assay was 2ng/mL.

The beta-estradiol was first dissolved in ethanol and then diluted further. It should be noted that the estradiol did not fully go into solution until a concentration of $2x10^{-4}$M was achieved. From there, a 10-fold dilution series was maintained and only 4µl of each dilution was used in a final assay volume of 1mL in order to keep the amount of ethanol to a minimum.

Assays:

The Promega Cell Titer 96 AQ Non-radioactive Cell Proliferation Assay was used to identify increases in proliferation rates for the MCF-7 cells. This was the primary assay used, although the Trevigen version of the same assay was used temporarily. The timeline took 50 hours from start to finish, with six distinct steps. At time 0, cells were plated at various concentrations in a 24 well plate in 1mL of 10% FBS DMEM. At 12 hours, the media was aspirated off and replaced with 1mL of Phenol-red free media (PHRED). At 24 hours, the media was aspirated off, and replaced with the appropriate combination of estradiol, IGF, and PHRED media, depending on the specific components being tested, up to a volume of 1mL. At 48 hours, the assay reagent was added to the well. Two hours later, at 50 hours, the plate was read at a wavelength of 450 nanometers.

Figure 3: Sample Assay Plate

Blank	Cells + 4µl IGF	Cells + 4µl estrogen	Cells +4µl IGF + 4µl estrogen	Cells + 4µl estrogen + 8µl IGF	Cells + 4µl more estrogen + 4µl IGF
Cells only	Cells + 4µl IGF	Cells + 4µl estrogen	Cells +4µl IGF + 4µl estrogen	Cells + 4µl estrogen + 8µl IGF	Cells + 4µl more estrogen + 4µl IGF
Media and 4µl estrogen	Cells + 4µl IGF	Cells + 4µl estrogen	Cells +4µl IGF + 4µl estrogen	Cells + 8µl IGF	Cells + 4µl less estrogen +4µl IGF
Media and 4µl IGF	Cells + 4µl IGF	Cells + 4µl estrogen	Cells +4µl IGF + 4µl estrogen	Cells + 8µl IGF	Cells + 4µl less estrogen +4µl IGF

Results

Assay Validation: Linearity with Cell Number

In order to validate the Promega Cell Titer Assay, cells were plated at 2 fold dilutions from $1.6x10^5$ to $4x10^4$ cells/well. The plate was then assayed as detailed in Methods. The data for cell number and the corresponding spectrophotometric readings are shown in Tables 1 and 2.

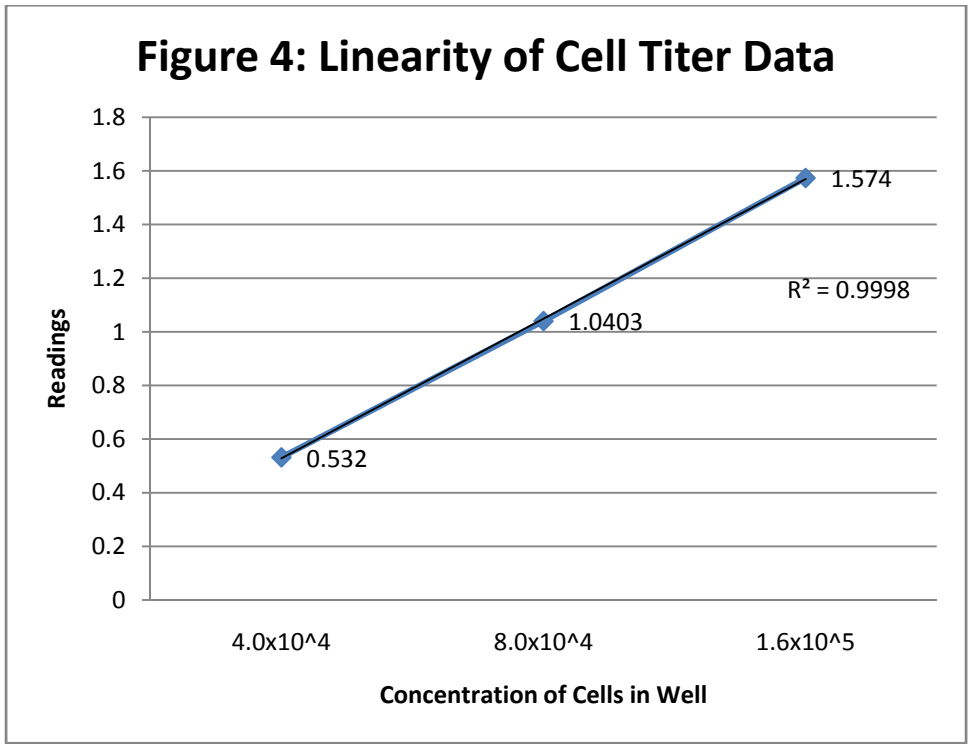
Table 1: Number of Cells Plated in Each Well of Assay Plate

	1	2	3	4	5	6
A	4.00E+04	4.00E+04	4.00E+04			
B	8.00E+04	8.00E+04	8.00E+04			
C	1.60E+05	1.60E+05	1.60E+05			
D	blank	blank	Control without assay	Control with assay	media	media

Table 2: Formazan Dye Concentration Readings at 450nm

	1	2	3	4	5	6	Avg. Readings	Avg. Minus Background
A	1.261	1.239	1.301				1.267	0.532
B	1.624	1.59	2.112				1.775	1.040
C	2.058	2.322	2.548				2.309	1.574
D	0.04	0.04	0.091	0.735	0.224	0.226		

These data are shown graphically in Figure 4.



Assay Validation: Linearity with Cell Number throughout Time Course

In order to validate the Promega Cell Titer Assay, cells were plated at 2 fold dilutions from $1.6x10^5$ to $4x10^4$ cells/well. The plate was then assayed as detailed in Methods using a time-course to change the media at 12hours, 24hours, and then 48hours (see Methods). This is the ideal time-line for the MTT assay as it allows growth of the cells. The data for cell number and the corresponding spectrophotometric readings are shown in Tables 3 and 4.

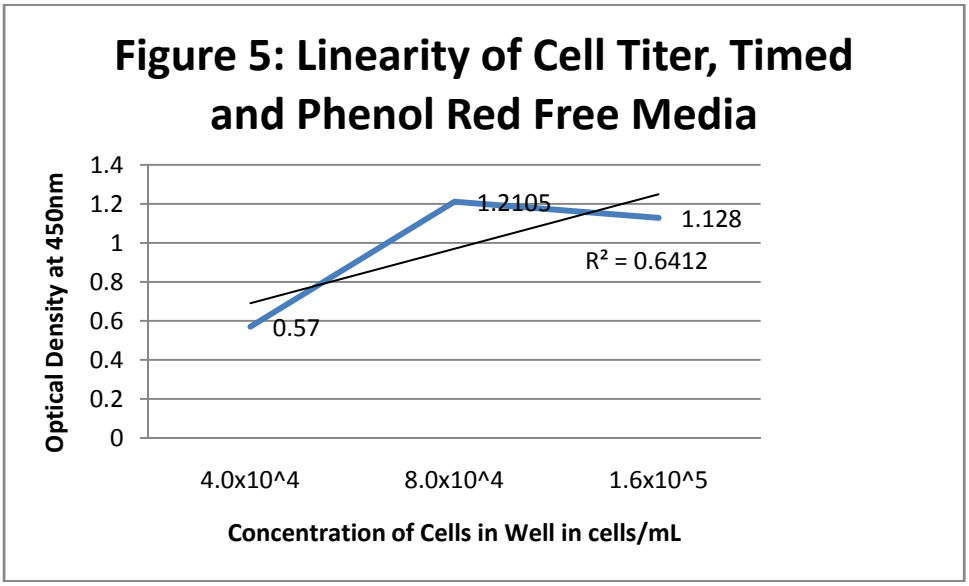
Table 3: Number of Cells Plated in Each Well of Assay Plate

	1	2	3	4	5	6
A	4.00E+04	4.00E+04				
B	8.00E+04	8.00E+04				
C	1.60E+05	1.60E+05				
D	blank	Control with Assay	Control without assay			

Table 4: Formazan Dye Concentration Readings at 450nm

	1	2	3	4	5	6	Avg. Readings	Avg. Minus Background
A	1.068	1.132					1.100	0.570
B	1.599	1.882					1.741	1.210
C	1.659	1.657					1.658	1.128
D	0.039	0.53	0.055					

These data are shown graphically in Figure 5:



Assay Validation: Comparison of MTT Cell Titer Kit with Comparison of Media

In this assay the Trevigen MTT Cell Titer Kit was used. The assay was designed to show comparability to the Promega Kit as well as comparing phenol red free media (PHRED) and DMEM. Cells were plated using 2 fold dilutions from $1.75x10^3$ to $4.00x10^4$ cells/well and assay performed as detailed in Methods. Table 5 shows the number of cells plated in each well while table 6 shows the spectrophotometric readings at 570nm.

Table 5: Number of Cells Plated in each Well of Assay Plate

	1	2	3	4	5	6
	DMEM	PHRED	PHRED	PHRED	PHRED	DMEM
A	5.00E+03	5.00E+03	5.00E+03	4.00E+04	4.00E+04	4.00E+04
B	2.50E+03	2.50E+03	2.50E+03	2.00E+04	2.00E+04	2.00E+04
C	1.75E+03	1.75E+03	1.75E+03	1.00E+04	1.00E+04	1.00E+04
D	DMEM contr +assay	control	control w/assay	blank	blank	DMEM contr w/o assay

Table 6: Formazan Dye Concentration Readings at 570nm							PHRED Avg. Rd.	DMEM Avg. Rd.
	1	2	3	4	5	6	0.017	0.119
	DMEM	PHRED	PHRED	PHRED	PHRED	DMEM	0.012	0.222
A	0.117	0.073	0.084	0.515	0.503	1.099	0.032	0.117
B	0.222	0.058	0.057	0.32	0.188	0.508	0.072	0.283
C	0.119	0.07	0.056	0.142	0.094	0.283	0.208	0.508
D	0.099	0.033	0.046	0.037	0.034	0.086	0.463	1.099

These data are shown graphically in Figures 6 and 7. Figure 6 shows the cell number versus dye reading for PHRED media alone, whereas Figure 7 combines both media.

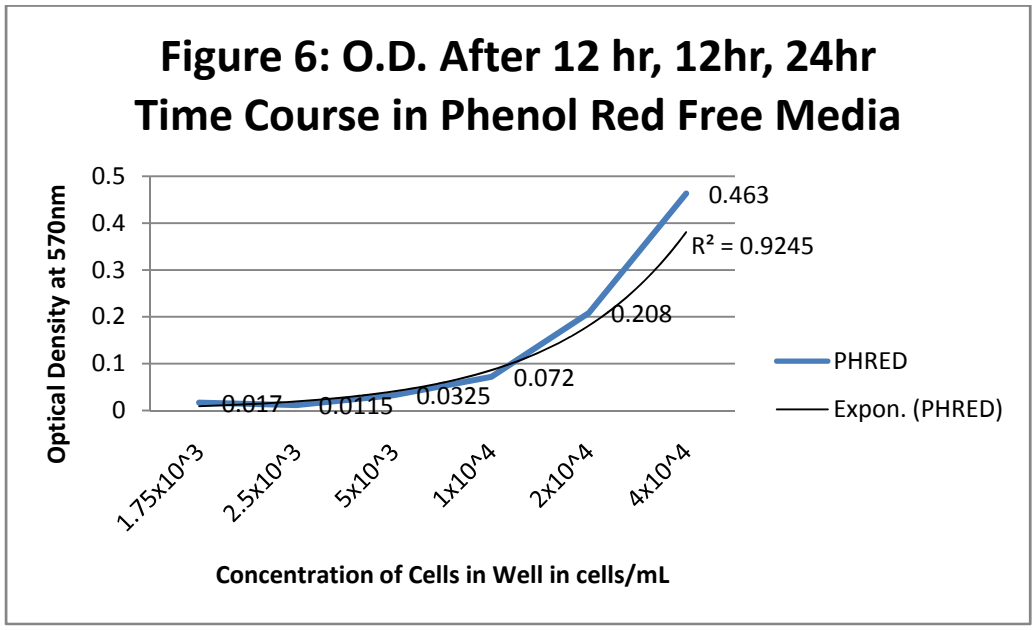
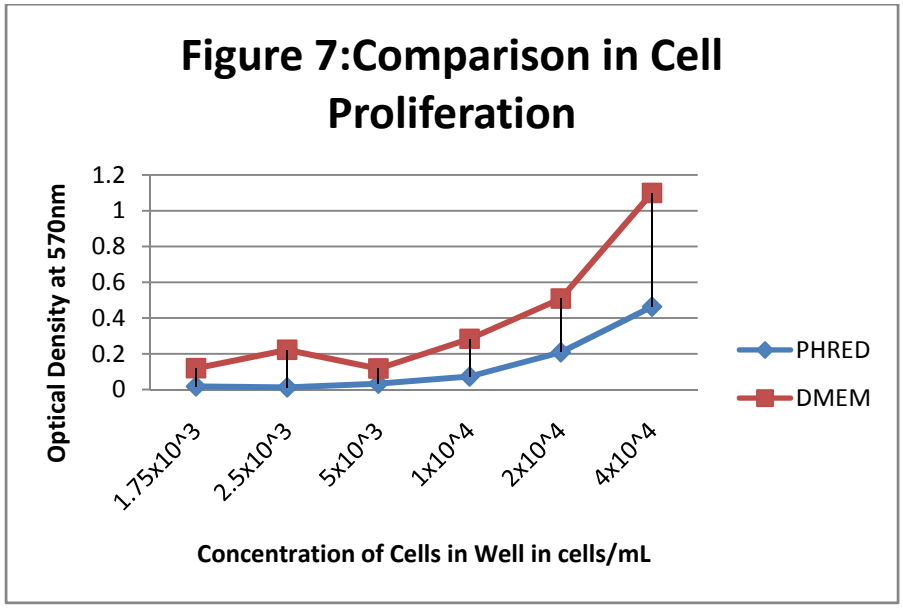


Figure 7 is a comparison of the cell proliferations readings with the different media. The red line is the DMEM readings, whereas the blue line is the phenol red free media.



Assay Validation: Control of Trevigen Assay Kit

In order to validate the Trevigen Cell Titer assay kit, a control experiment was set-up. Cells were plated at $2.0x10^4$ cells/well and combinations of cells, estrogen, ethanol, and assay reagent were added as detailed in Methods. The data for the plate-setup and spectrophotometric readings are shown in tables 7 and 8.

Table 7:Variations in Cells, Estrogen, and Assay Reagent in Wells

	1	2	3	4	5	6
A	Cells	Cells	Cells + EtOH	Cells + EtOH	Cells+EtOH+E2	Cells+EtOH+E2
B	Cells	Cells	Cells + EtOH	Cells + EtOH	Cells+EtOH+E2	Cells+EtOH+E2
C	Cells No Assay	Cells No Assay	Cells + EtOH No Assay	Cells + EtOH No Assay	Cells+EtOH+E2 No Assay	Cells+EtOH+E2 No Assay
D	blank	blank	Control	Control + EtOH Assay	Control	Control + EtOH+E2 Assay

The no assay wells had no color change, and the cells were observed to be alive. The cells in the other wells, after adding that assay and detergent, looked as if crystals had developed. They were no longer round, and were dead.

Table 8: Formazan Dye Readings at 570nm

	1	2	3	4	5	6
A	0.193	0.256	0.178	0.214	0.105	0.094
B	0.161	0.186	0.173	0.171	0.119	0.09
C	0.036	0.037	0.039	0.039	0.063	0.098
D	0.031	0.029	0.041	0.042	0.071	0.072

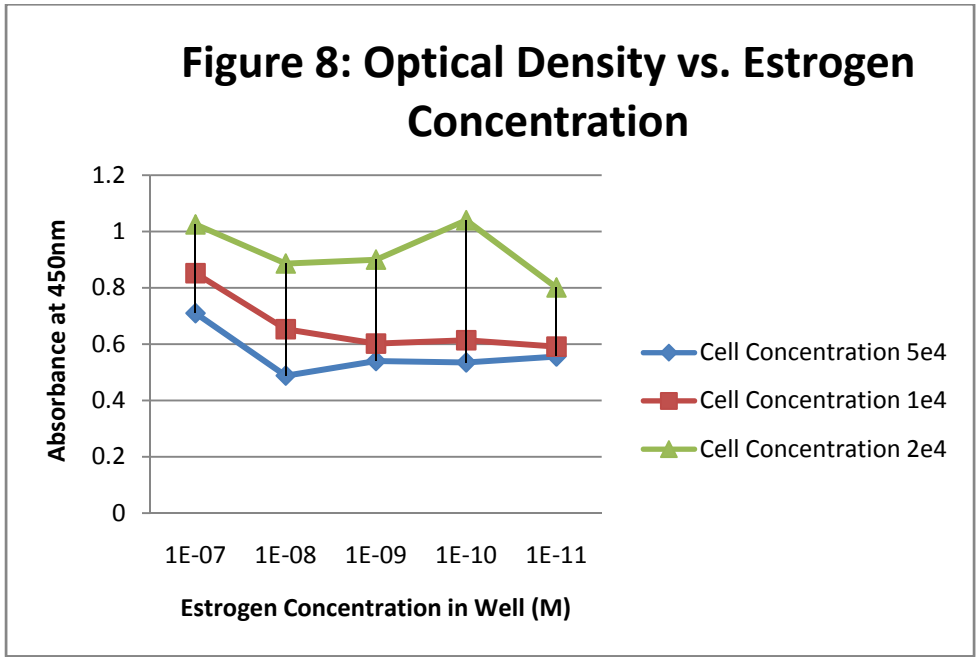
Estrogen Level Variation Assay:

The following data shows the results of the Promega MTT Cell Titer Assay with variations of estrogen concentration. Granular 17β-Estradiol was dissolved in ethanol as described in Methods, and the appropriate concentrations from $1.00x10^{-5}$ to $1.00x10^{-9}$M were added to the plate. The amount of estrogen by cell number is shown in Table 9, while the spectrophotometric readings at 450nm are shown in Table 10.

Table 9: Estrogen Concentration and Number of Cells in Assay Plate							
		Estrogen Level					
Cell #	1	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	
5.00E+03	Blank	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	450
1.00E+04	Cells	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	450
2.00E+04	Cells+Assay	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	450
D	Media	Media+Assay	Media+Ethanol+Assay	Cells+Ethanol	Media+Ethanol+Estrogen	Media+Ethanol+Estrogen+Assay	450

Table 10: Formazan Dye Readings at 450nm							
	1	2	3	4	5	6	
A	0.039	0.710	0.488	0.540	0.535	0.556	450
B	0.059	0.852	0.653	0.602	0.614	0.591	450
C	0.993	1.025	0.886	0.900	1.040	0.801	450
D	0.050	0.498	0.310	0.068	0.301	0.591	450

These data are shown graphically in Figure 8, showing each cell number with respective estrogen level and dye reading.



Estrogen Level Variation Assay 2:

The following data shows the results of the Promega MTT Cell Titer Assay with variations of estrogen concentration. Granular 17β-Estradiol was dissolved in ethanol as described in Methods, and the appropriate concentrations from $1.00x10^{-7}$ to $1.00x10^{-11}$M were added to the plate. The amount of estrogen by cell number is shown in Table 11, while the spectrophotometric readings at 450nm are shown in Table 12.

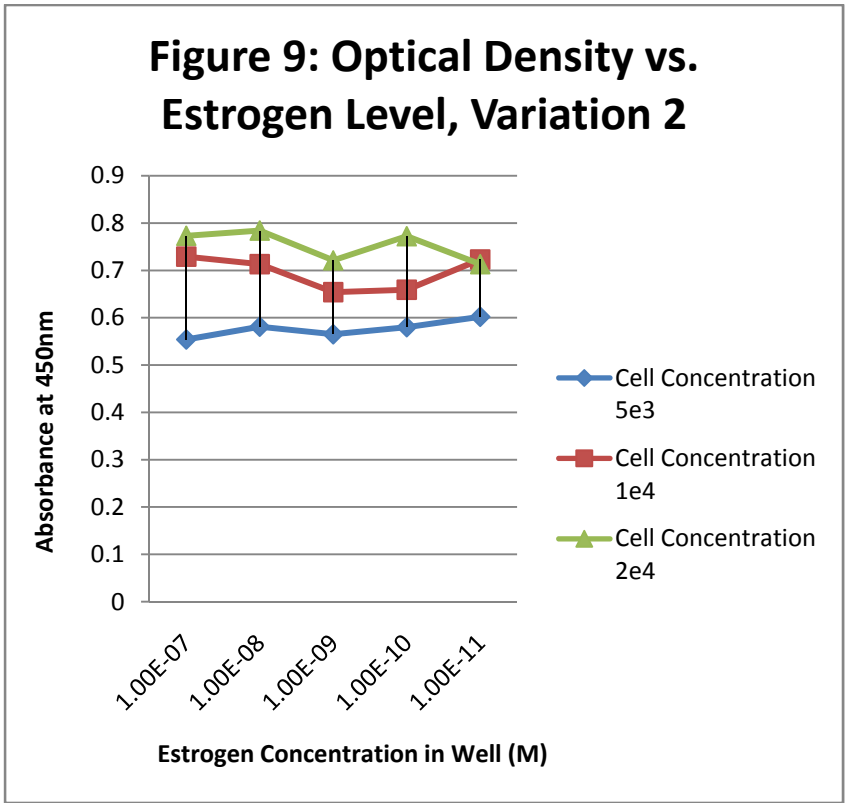
Table 11: Estrogen Level by Cell Number added to Assay Plate

		Estrogen Level					
Cell #	1	1.00e-07	1.00E-08	1.00E-09	1.00E-10	1.00E-11	
5.00E+03	Blank	0.554	0.581	0.565	0.580	0.602	450
1.00E+04	Cells	0.729	0.713	0.654	0.659	0.723	450
2.00E+04	Cells+ Assay	0.773	0.784	0.721	0.772	0.713	450
D	Media	Media+ Assay	Media+Ethanol+Assay	Cells+Ethanol	Media+Ethanol +Estrogen	Media+Ethanol+Estrogen+Assay	450

Table 12: Formazan Dye Reading at 450nm

	1	2	3	4	5	6	
A	0.038	0.554	0.581	0.565	0.580	0.602	450
B	0.056	0.729	0.713	0.654	0.659	0.723	450
C	0.905	0.773	0.784	0.721	0.772	0.713	450
D	0.052	0.051	0.554	0.061	0.058	0.543	450

These data are shown graphically in Figure 9, showing each cell number with respective estrogen level and dye reading.



Addition of Insulin-Growth Factor-II (IGF-II) Assay

In order to induce cell proliferation, IGF-II was added to the plate once cells were plated at levels from $2.00x10^4$ to $5.00x10^3$ cells/well. Varying estrogen levels were added to the wells starting at $1.00x10^{-5}$ to $1.00x10^{-9}$M, and IGF-II was added at a concentration of 2ng/mL. The number of cells, levels of estrogen, and IGF well locations are shown in Table 13, and spectrophotometric readings at 450nm are shown in Table 14.

Table 13: Number of Cells, Estrogen Level, and Location of IGF-II

		Estrogen Level					
Cell #	1	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	
5.00E+03	Blank	Estrogen+IGF	Estrogen+IGF	Estrogen+IGF	Estrogen+IGF	Estrogen+IGF	450
1.00E+04	Cells	Estrogen+IGF	Estrogen+IGF	Estrogen+IGF	Estrogen+IGF	Estrogen+IGF	450
2.00E+04	Cells+Assay	Estrogen+IGF	Estrogen+IGF	Estrogen+IGF	Estrogen+IGF	Estrogen+IGF	450
D	Media	Media+Assay	Media+Ethanol+Assay	Cells+Ethanol	Media+Ethanol+Estrogen	Media+Ethanol+Estrogen+Assay	450

Table 14: Formazan Dye Concentration at 450nm after 1 hr.

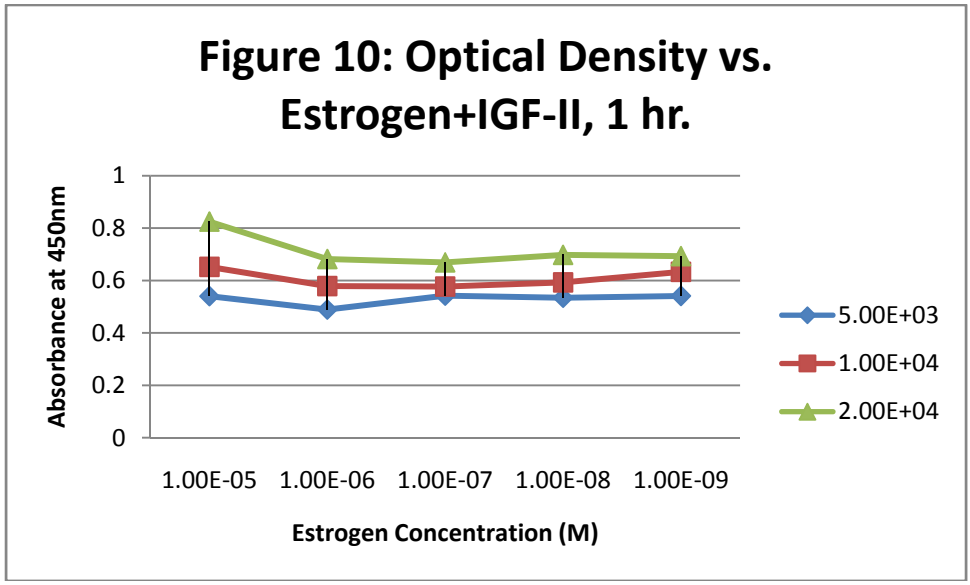
	1	2	3	4	5	6	
A	0.041	0.54	0.489	0.542	0.534	0.541	450
B	0.052	0.652	0.579	0.577	0.593	0.633	450
C	0.622	0.825	0.682	0.669	0.698	0.693	450
D	0.054	0.503	0.508	0.059	0.111	0.669	450

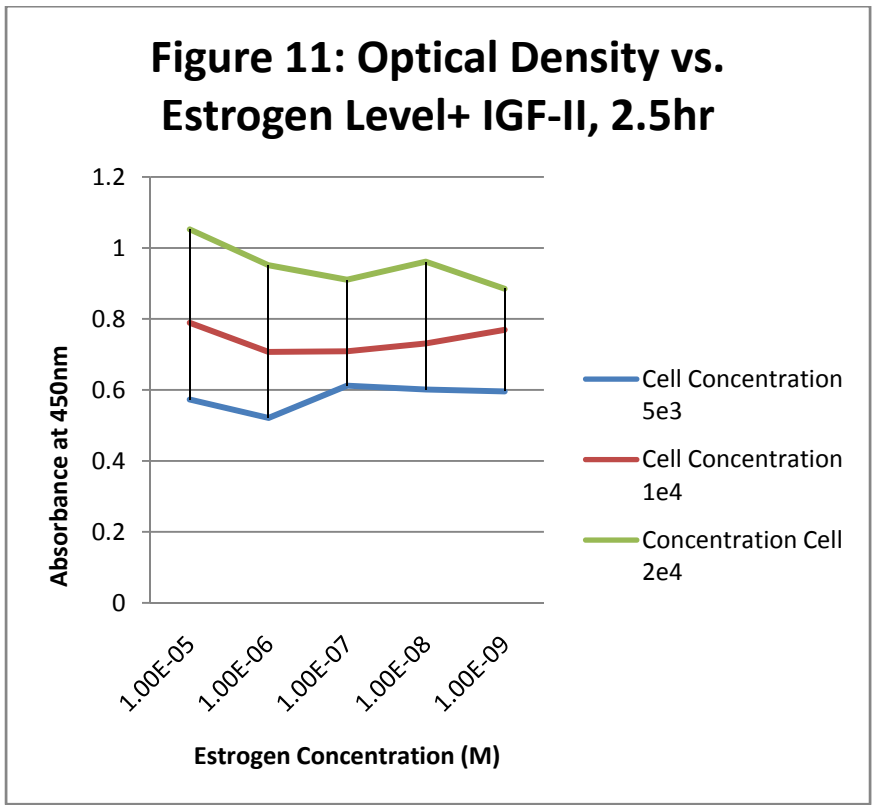
The assay reagent was given approximately 1 hour in order to form the formazan dye complex. This was noted in the assay materials. However, it may take up to four hours for the complex to develop. Table 15 shows the concentrations 2.5hours after adding reagents.

Table 15: Formazan Dye Concentration at 450nm after 2.5hrs

	1	2	3	4	5	6	
A	0.042	0.573	0.521	0.612	0.601	0.596	450
B	0.052	0.789	0.707	0.709	0.731	0.769	450
C	0.843	1.052	0.951	0.91	0.961	0.885	450
D	0.054	0.539	0.542	0.059	0.116	0.68	450

These data are shown graphically in Figures 10 and 11. Figure 11 shows the estrogen level with IGF-II after 1 hour, and Figure 12 shows estrogen with IGF-II at 2.5hrs.





Variation in Estrogen Level, IGF-II, and Cell Number Assay

In order to determine the effects of estrogen and IGF on the cells, variations in estrogen level, IGF concentration, and cell number were varied in this assay. The amount of IGF added to the wells was 2ng/mL and 4ng/mL, with one set of wells with no IGF. The estrogens levels used were $2x10^{-3}$M in plate A, and $2x10^{-4}$M, for final concentrations in the well of $1x10^{-5}$M and $1x10^{-6}$M respectively. The two different concentrations of cells used were $2x10^{4}$cells/mL and $4x10^{4}$cells/mL. Table 18 shows the results from Plate A, Table 19 are the results from Plate B, and Tables 16 and 17 show how Plate A and B were set-up respectively.

Table 16: Variations of Estrogen, Cell Number, and IGF on Well Plate

		Estrogen Level = 2x10^-3					
Plate A	1	2	3	4	5	6	
A	Plate A = Blank	No IGF	2ng/mL IGF	2ng/mL IGF	4ng/mL IGF	4ng/mL IGF	Cocentration = 2x10^4 Cells
B	Media+Ethanol+Assay	NO IGF	2ng/mL IGF	2ng/mL IGF	4ng/mL IGF	4ng/mL IGF	
C	Media+E2+Assay	NO IGF	2ng/mL IGF	2ng/mL IGF	4ng/mL IGF	4ng/mL IGF	Concentration = 4x10^4 cells
D	Media+Ethanol+Acetic+BSA+Assay	NO IGF	2ng/mL IGF	2ng/mL IGF	4ng/mL IGF	4ng/mL IGF	

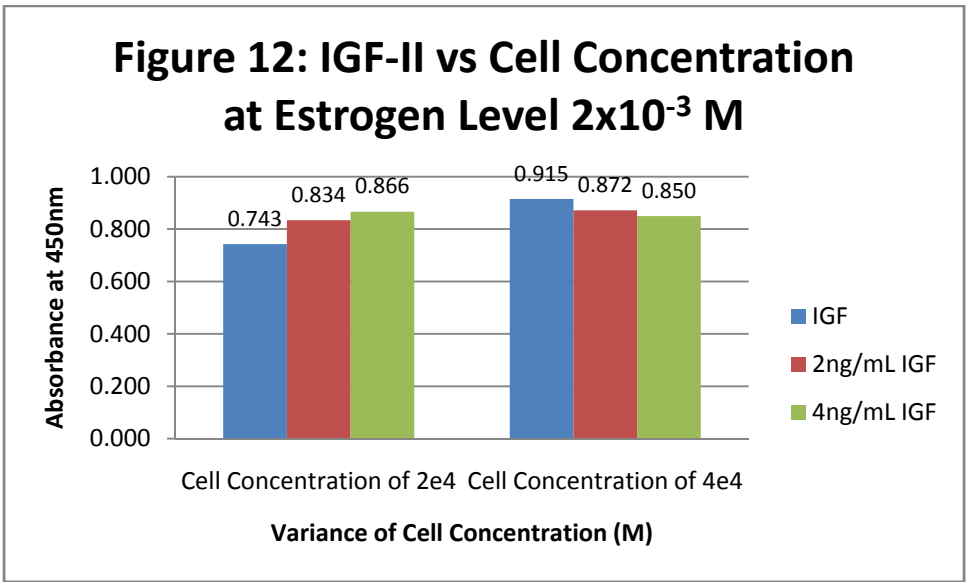
Table 17: Variations of Estrogen, Cell Number, and IGF on Well Plate

		Estrogen Level = 2x10^-4					
Plate B	1	2	3	4	5	6	
A	Media+E2+IGF+Assay	No IGF	2ng/mL IGF	2ng/mL IGF	4ng/mL IGF	4ng/mL IGF	Cocentration = 2x10^4 Cells
B	Media+Cells+Assay	NO IGF	2ng/mL IGF	2ng/mL IGF	4ng/mL IGF	4ng/mL IGF	
C	Media+Cells+Ethanol+Assay	NO IGF	2ng/mL IGF	2ng/mL IGF	4ng/mL IGF	4ng/mL IGF	Concentration= 4x10^4 cells
D	Media+Cells+Ethanol+Acetic Acid +BSA+assay	NO IGF	2ng/mL IGF	2ng/mL IGF	4ng/mL IGF	4ng/mL IGF	

Table 18: Plate A Formazan Dye Concentration after 1hr.							
Plate A	1	2	3	4	5	6	
A	0.039	0.703	0.851	0.887	0.69	0.993	450
B	0.494	0.783	0.858	0.741	0.801	0.981	450
C	0.538	1.038	0.943	1.006	0.748	0.794	450
D	0.522	0.792	0.737	0.802	0.97	0.89	450

Table 19: Plate B Formazan Dye Concentration after 2.5hrs							
Plate B	1	2	3	4	5	6	
A	0.555	0.669	0.539	0.611	0.575	0.595	450
B	0.648	0.67	0.616	0.61	0.601	0.588	450
C	0.704	0.794	0.725	0.692	0.694	0.643	450
D	0.723	0.783	0.69	0.647	0.587	0.64	450

These data are shown graphically in Figures 12 and 13. Figure 12 shows the levels of IGF-II versus cell concentration at estrogen level of $2x10^{-3}$M. Figure 13 shows the levels of IGF-II versus cell concentration at estrogen level of $2x10^{-4}$M.



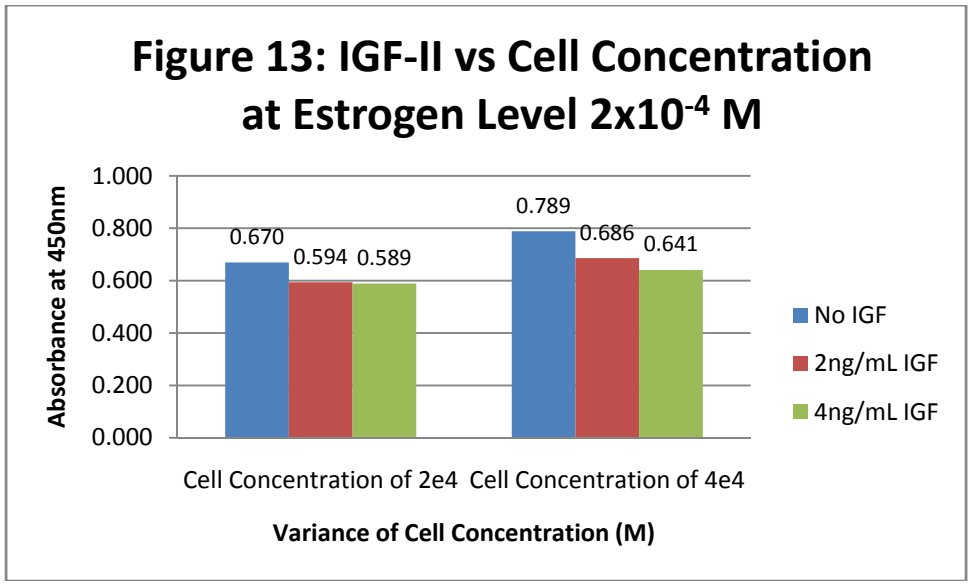


Figure 13: IGF-II vs Cell Concentration at Estrogen Level 2x10-4 M
Absorbance at 450nm
1.000
0.800
0.600
0.400
0.200
0.000
0.670
0.594
0.589
0.789
0.686
0.641
No IGF
2ng/mL IGF
4ng/mL IGF
Cell Concentration of 2e4
Cell Concentration of 4e4
Variance of Cell Concentration (M)

Variation in Estrogen Level and IGF-I Assay:

In order to test estrogen level and IGF-I, the MTT assay was performed as detailed in Methods. This assay was performed after making fresh stock of IGF-1, estrogen stocks, and cells at a lower passage of 14. The data shows the results of varying estrogen levels and IGF added to the cells. The cell number stayed constant in each well at $2x10^4$ cells/well. IGF concentrations used were 2ng/ml and 4ng/ml. The amount of estrogen used was $2x10^{-4}$M, with more being $2x10^{-3}$M, and lesser amounts being $2x10^{-5}$M and $2x10^{-7}$M. Table 21 shows the spectrophotometric readings while Table 20 shows how the plate was set-up.

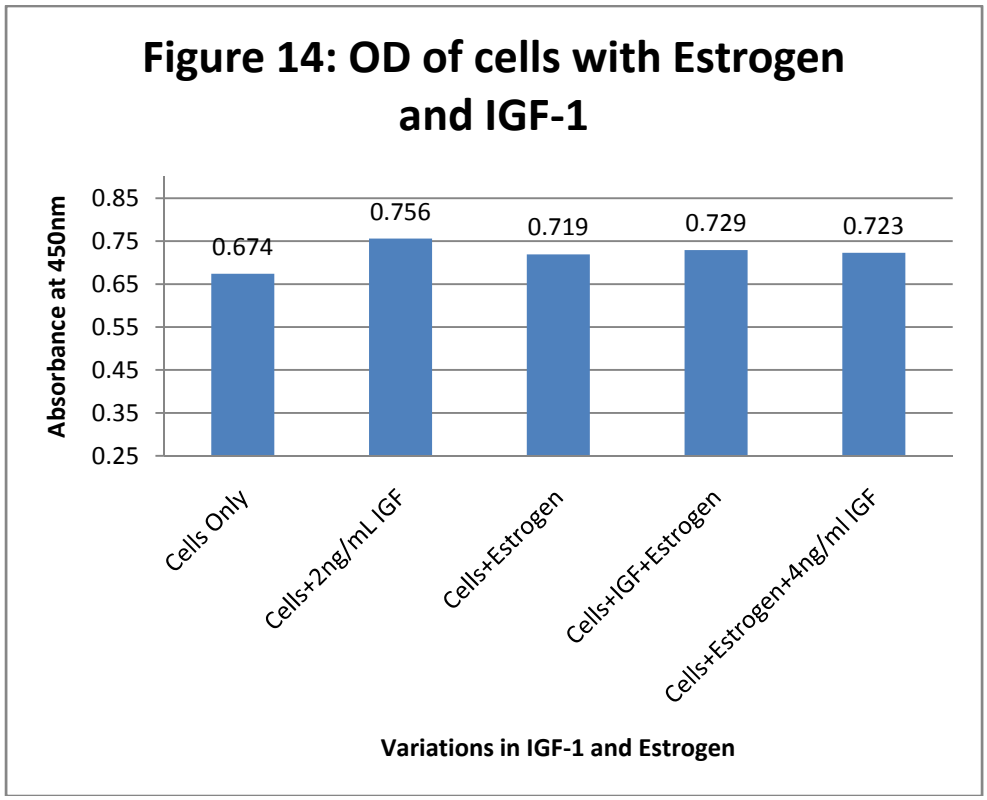
Table 20: Variation in IGF and Estrogen Concentration

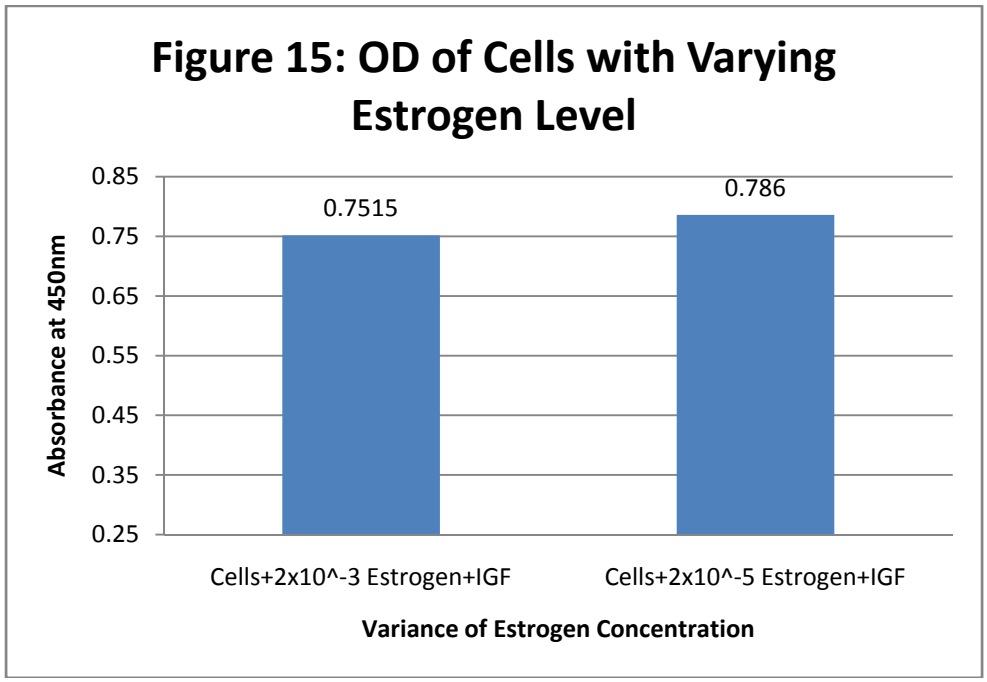
	Controls	Cells+IGF	Cells+Estrogen	Cells+IGF+Estrogen	Varied IGF	Cells+2x10^-3 Estrogen+IGF	
A	Blank	Cells+IGF	Cells+Estrogen	Cells+IGF+Estrogen	Cells+Estrogen+4ng/mL IGF	Cells+2x10^-3 Estrogen+IGF	450
B	Cells Only	Cells+IGF	Cells+Estrogen	Cells+IGF+Estrogen	Cells+Estrogen+4ng/mL IGF	Cells+2x10^-3 Estrogen+IGF	450
C	Media and Estrogen	Cells+IGF	Cells+Estrogen	Cells+IGF+Estrogen	Cells+4ng/mL IGF	Cells+2x10^-5 Estrogen+IGF	450
D	Media and IGF	Cells+IGF	Cells+Estrogen	Cells+IGF+Estrogen	Cells+4ng/mL IGF	Cells+2x10^-5 Estrogen+IGF	450

Table 21: Formazan Dye Concentration at 450nm

	1	2	3	4	5	6	
A	0.039	0.664	0.726	0.765	0.723	0.741	450
B	0.674	0.828	0.733	0.739	0.723	0.762	450
C	0.537	0.832	0.68	0.694	0.721	0.823	450
D	0.552	0.7	0.738	0.719	0.754	0.749	450

These data are shown graphically in Figures 14 and 15. Figure 14 shows the variations in each condition versus optical density. Figure 15 shows the variations in estrogen level showing the optical density with more and less estrogen.





Discussion

The MCF-7 cells used in this project were found to be unresponsive to estrogen, which prohibited the study of phytoestrogens. In order to study any effects on proliferation rates, there would need to be a baseline of proliferation that was obtained in the presence of estrogen. Ideally the estrogen would have mimicked both the traditional hormone replacement therapy and the natural presence of estrogen in the body. Without identifying this baseline, the research could not progress.

A series of nine assays were performed throughout the project, and the purpose of the first assay was to determine what concentration of cells would be most appropriate for the later assays. This assay was performed in regular DMEM and without the strict timeline. The cells were plated at concentrations of $4.0x10^4$, $8.0x10^4$, and $1.6x10^5$ cells/mL. After being allowed to grow for 24 hours, the assay was added to the wells with cells as well as the control well containing only media. This was used to determine the amount of background absorbance. After averaging the absorbance readings for each cell concentration and subtracting the average background absorbance, as seen in Figure 4, the absorbance can be plotted against cell concentration to find the linearity. Assay 1 did not reach the upper or lower limit of cell concentration, so further concentrations of cells needed to be pursued. This assay did, however, show that the assay worked properly and could be used on later assays. In the next attempt to establish the upper and lower limits of the cell concentration, a structured timeline was used to imitate the conditions of a true assay. This was also the first assay where PHRED was used. Cells were plated down at the same concentrations as the first assay, with the understanding that they would have a longer amount of time to grow in the wells, and therefore may reach their upper limit of growth. 12 hours after being plated, the media was changed from DMEM with 10% FBS to the PHRED media. 24 hours after this initial media switch, the media was changed once more to simulate the addition of reagents. 12 hours later the assay was added to the plate in the appropriate wells and the plate was read 1 hour later. After subtracting the background absorbance, the readings were plotted, revealing an upper limit to the cell concentration. Since there was very little change in absorbance readings between $8.0x10^4$ and $1.6x10^5$ cells/mL in Figure 5, this was established as the upper limit for cell line and well size being used for this assay.

For the third assay, the assay kit being used was switched to the Trevigen kit for price difference reasons. The kit functions the same way and only difference is the addition of a detergent before reading the assay. For this assay, the timeline was also adjusted to a 12-12-24 hour timeline in order to properly reflect the steps of plating the well, changing to PHRED, adding estrogen, and then adding assay. The main purpose of the third assay was to determine the lower limit of the cell concentration as well as determine the validity of using PHRED instead of DMEM. Table 6 shows the layout of the plate, split so that each cell concentration

was grown in either the DMEM the whole time or put into PHRED at the appropriate time. Figure 6 depicts the absorbance readings for only the cells grown in PHRED, indicating that the lower limit for the cells was between $5.0x10^3$ and $1.0x10^4$ cells/mL. $1.0x10^4$ was chosen as the ideal cell concentration for the subsequent assays, as this would allow the cells to proliferate during the timeframe of the assay without hitting their upper limit. Figure 7 clearly shows the difference in growth rates for the cells kept in DMEM for the entirety of the assay. While the growth lines increase in a similar fashion, the cells grown in DMEM have higher growth rates at each concentration, despite the fact that they were plated at the same concentration. This effect is likely due to the presence of phenol-red in the DMEM. Phenol-red interacts with the estrogen receptor in a manner similar to estrogen, although at a greatly reduced efficiency. Therefore eliminating the phenol-red from the media as is done in the PHRED media, any possibility of false positives due to the phenol-red can be eliminated.

The intention of the fourth assay was to begin looking at the effects of estrogen on the cells to serve as a basis of comparison against phytoestrogens. However, the Trevigen assay kit began causing some problems. Two separate assays were attempted (data not shown), so the goal of the next attempt, detailed in Table 7, was to determine if it was the assay, the estrogen, the ethanol, or the cells that was causing the problem. In the previous attempts, no color change was observed and the readings were all on the same level as the blank. When looking at the cells by the end of the assay, they appeared to have crystal like structures attached to them. After reading the plate, it was decided that the assay was at fault, based on the low readings across the board, and it was decided that the 6 weeks since purchasing the assay had probably lead to the degradation.

Due to the rapid degradation of the assay from Trevigen, the assay kit was switched back to Promega. After having established the proper timeline and ideal cell concentration, estrogen was added to the assay to begin simulating the estrogen found in hormone replacement therapy. At this point it was assumed that the cells were estrogen responsive, as advertised by the ATCC, but the concentration of estrogen needed to elicit a response was still unknown. Table 9 shows the absorbance readings for the fifth assay, in which the cell concentrations were $5.0x10^3$, $1.0x10^4$, and $2.0x10^4$cells/mL. These concentrations were varied because it was still unknown how the cells would respond in the presence of estrogen. The estrogen concentrations ranged from $1x10^{-5}$ to $1x10^{-9}$M in ten-fold dilution steps. Previous MQPs had used a concentration of $1x10^{-9}$M, but this assay wanted to find if there was any response at all before narrowing it down to one single concentration. This was the first successful assay where the cells were grown in the presence of estrogen. The absorbance readings, however, showed little difference in growth rate when any amount of estrogen was added. In the physical observations of the wells before reading the plate, the wells with the highest concentration of estrogen appeared grainy in the spaces between the cells. It was hypothesized that the estrogen may be coming out of solution at that high of a concentration.

The 6th assay looked a lower range of estrogen concentrations, while still varying the cell concentration from $5x10^3$ to $2x10^4$ cells/mL. Similar to the 5th assay, there was little difference across the range of estrogen doses, and the only apparent increases occurred because of the cell concentration increase. This simply showed that more cells do lead to higher absorbance readings at the end of the assay, but these increases were due to normal cell growth. After the 5th and 6th assays were completed, there was no clear concentration at which the addition of estrogen resulted in an increase in cell proliferation, despite the cells' supposed estrogen responsiveness.

In an effort to amplify the cells' growth response to estrogen, Insulin-like Growth Factor was incorporated into the assay. Hamlers *et al.* showed that the addition of IGF can amplify the proliferation response to estrogen. The 7th assay included IGF-II at 2ng/mL in all wells where estrogen was added. Figures 10 and 11, however, show that there was no significant increase in cell proliferation rates. The variation in cell concentration at the original plating was the only variable to affect the absorbance readings, and the range of estrogen concentrations depicted in Table 15 showed no affect.

The 8th assay made each of the reagents a variable to see if there was any optimal combination that could result in an increase in cell proliferation. It was thought that the cells may still respond to estrogen, but at a particular combination or concentration what was not yet being utilized. IGF-II was added at both 2ng/mL and 4ng/mL. Estrogen was added at $2x10^{-3}$M and $2x10^{-4}$M. Cells were plated at $2x10^4$ and $4x10^4$ cells/mL. A no IGF-II control was also used. Each well was performed in duplicate, to reduce the chance of extreme variation. However, no major increases were seen in any of the combinations, as seen in Figures 12 and 13.In the final assay, the plate used the maximal amounts of reagents. A brand new stock of IGF-I was used, since it had been studied further than IGF-II and had successfully shown an increase in growth rates when used in combination with estrogen. The final plate included wells that had both 2ng/mL and 4ng/mL of IGF-I. For estrogen, a brand new stock with new dilutions was used. The majority of the cells received $1x10^{-6}$M, with some wells using ten-fold more or ten-fold less. The same concentration of cells, $2x10^4$ cells/mL, was used in every well, but a lower passage was used. Passage 14 cells were used because it was the closest available passage to the Passage 17 cells that had shown an increase in the presence of Phenol-red. Previous to this assay, the cells had grown up to passage 35, and it was suspected that they may have lost their estrogen-responsiveness. However, the assay results showed no major differences in proliferation rates, as depicted in Figures 14 and 15.

Despite its inefficacy, the idea behind using a lower passage number was still an important one. It is believed that when grown in the absence of estrogen for prolonged amounts of time, MCF-7 cells can lose their estrogen responsiveness. When not being assayed, the cells used in this assay were not supplied with estrogen, and therefore spent roughly 6 months growing without estrogen. It is possible that the continued passaging selected for a

sub-population of cells that may have lacked the estrogen receptor but could grow successfully without it. While the data from the last assay suggests that this was not the case, it is important to note that the original stock of cells were obtained already at passage 7 and the Phenol-red may have affected the cells in a pathway unrelated to the estrogen receptor. The cells were passaged by someone other than this project group for 7 passages after being obtained from the ATCC. It is also possible that selection occurred in those first 7 passages that eliminated the estrogen-responsiveness of the cells. When initially isolated from a breast carcinoma, the heterogeneous cell line consisted of several different subpopulations that could easily have been selected for based on the initial growth conditions.

Several things became apparent as a result of all of these assays. This stock of MCF-7 cells is definitely not responsive to estrogen. After testing with IGF-I and II, as wells a numerous different concentrations of estrogen, the cells showed no increase or decrease in cell proliferation rates. In further studies it would be worthwhile to use alternative stocks of estrogen-responsive breast epithelial cells, such as T-47D or ZR-75-1, which are both available through the ATCC The cells did grow more in the Phenol-red media, but it is difficult to pinpoint the cause of this response. It had been thought to be passage number, but our final assay ruled that out. The Phenol-red may have been interacting with the cell through a pathway other than the estrogen receptor.

The possibility of other cell lines contaminating the MCF-7 cultures had been ruled out because no other cell lines were used in the same hood or the same incubator. Some equipment was shared with sp2 cells and L6 cells, but it is unlikely that any contamination occurred. However, it again becomes important to note that these cells were obtained at passage 7, and it is impossible to know the exact details of their matainence before being frozen. It is also important to consider the risk involved in assuming certain cell lines possess certain characteristics. Determining estrogen responsiveness earlier on in the project could have provided enough time to switch cells lines. For future experiments, it would be worth adding estrogen to a select number of wells in the early assays to look for any kind of response before moving forward. Performing a western blot for the estrogen receptor would also be another way of looking into estrogen responsiveness. It would be interesting to see if the lack of estrogen responsiveness was due to a lack of estrogen receptor, and if so, how did that change over time with passage number. The same could be performed for both IGF-I and II.

IGF-I and II cannot be ruled out as affecting cell proliferation rates. It is already known that these growth factors are modulated through the estrogen-receptor pathway, and it is highly possible that they may have an effect on an estrogen responsive cell line. Increased expression of IGF-II has been seen in higher passages of MCF-7 cells, and it is thought that this may be responsible for the successful growth of the cells, as cells expressing an autocrine growth factor could out-compete cells that do not express it. Starting with IGF-I may yield positive results, but following up with IGF-II could provide novel insight into the pathway.

Ideally, after determining estrogen responsiveness in a cell line, this estrogen baseline could be compared to the effects on proliferation from phytoestrogens. Originally, this project had planned on using Genistein, a phytoestrogen found in Promensil, an over-the-counter treatment for menopausal women. After learning the effects of that specific phyotoestrogen, other compounds in over-the-counter treatments such as Promensil could be looked at, alone or in combination with the phyotestrogens. There is much to be studied in this topic, but it is all dependent on finding an estrogen-responsive cell line.

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