The Effects of Selenium on Estrogen-regulated Gene Expression in LNCaP Prostate Cancer Cells

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THE EFFECTS OF SELENIUM ON ESTROGEN-REGULATED GENE EXPRESSION
IN LNCAP PROSTATE CANCER CELLS

By
Tory L. Parker

A thesis submitted to the faculty of
Brigham Young University
In partial fulfillment of the requirements for the degree of

Master of Science

Department of Nutrition, Dietetics and Food Science
Brigham Young University
December 2004
Of a thesis submitted by

Tory L. Parker

This dissertation has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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As chair of the candidate's graduate committee, I have read the thesis of Tory L. Parker in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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Accepted for the college

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THE EFFECTS OF SELENIUM ON ESTROGEN-REGULATED GENE EXPRESSION IN LNCAP PROSTATE CANCER CELLS

Tory L. Parker

Department of Nutrition, Dietetics and Food Science

Master of Science

Prostate cancer is the most frequently diagnosed cancer in American men and the second leading cause of cancer deaths. Supplementation with Se has reduced the incidence of prostate cancer and Se status is inversely correlated with prostate cancer risk. One molecular mechanism by which high Se concentrations may affect cancer risk is by catalyzing disulfide bond formation or otherwise complexing with reactive sulfhydryl groups in cellular proteins. The estrogen receptor (ER) contains cysteines in zinc (Zn) fingers that are susceptible to oxidation and internal disulfide formation, which can prevent DNA binding. We examined ER binding to its DNA response element and gene expression levels for estrogen-regulated genes in human prostate cancer cells (LNCaP) treated with control (50 nM) or high (5 µM) concentrations of Se. High Se treatment resulted in a non-significant 16 % decrease in ER binding to the estrogen response element (ERE), and no significant changes were found in expression levels of
estrogen-regulated genes for either run-on nuclear transcripts or total mRNA. The well documented reaction of Se with reactive sulphydryl groups, if it occurs in the ER *in vivo*, has a minimal effect on the binding of ER to DNA and its regulation of gene expression.
ACKNOWLEDGEMENTS

I would like to thank my committee members for their input and suggestions, especially Dr. Merrill Christensen. Appreciation is noted for Josie Johnson, Jake Majors and Dr. Dennis Eggett, who greatly assisted with the experimentation process and statistical analysis. Most of all, I would like to thank my wife, Andrea, for her encouragement and patience during the many months of late night returns home.
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Original Research

The Effects of Selenium on Estrogen-Regulated Gene Expression

in LNCaP Prostate Cancer Cells

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Running head: Effects of selenium on estrogen-regulated genes

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ABSTRACT

Prostate cancer is the most frequently diagnosed cancer in American men and the second leading cause of cancer deaths. Supplementation with Se has reduced the incidence of prostate cancer and Se status is inversely correlated with prostate cancer risk. One molecular mechanism by which high Se concentrations may affect cancer risk is by catalyzing disulfide bond formation or otherwise complexing with reactive sulfhydryl groups in cellular proteins. The estrogen receptor (ER) contains cysteines in zinc (Zn) fingers that are susceptible to oxidation and internal disulfide formation, which can prevent DNA binding. We examined ER binding to its DNA response element and gene expression levels for estrogen-regulated genes in human prostate cancer cells (LNCaP) treated with control (50 nM) or high (5 µM) concentrations of Se. High Se treatment resulted in a non-significant 16 % decrease in ER binding to the estrogen response element (ERE), and no significant changes were found in expression levels of estrogen-regulated genes for either run-on nuclear transcripts or total mRNA. The well documented reaction of Se with reactive sulfhydryl groups, if it occurs in the ER in vivo, has a minimal effect on the binding of ER to DNA and its regulation of gene expression.
INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer in American men and the second leading cause of cancer deaths. The American Cancer Society estimates that prostate cancer will account for 33% of new cancers in men, totaling over 230,000 new cases, with 29,900 deaths in 2004 (39). Supplementation with 200 µg of Se in the trials of Clark et al. reduced the incidence of prostate cancer (4). The most recent analysis of their data showed the greatest benefit from Se supplementation in men in the lowest two tertiles of baseline serum Se (7). High Se status has been correlated with reduced prostate cancer risk (44, 45, 48) and may slow the progression of prostate cancer tumors (29). Dietary supplementation with Se compounds may represent a viable option to reduce incidence, morbidity, and mortality of prostate cancer (31).

The mechanisms for Se's protective effects have not been fully explored, though many possible molecular targets have been identified (13, 23, 38, 47). Ganther (8, 9) proposed that one molecular mechanism by which high Se concentrations may affect cancer risk is by catalyzing disulfide bond formation or otherwise complexing with reactive sulfhydryl groups in cellular proteins. Formation of such structures has been demonstrated in catalytic proteins (11). Our laboratory has recently provided evidence consistent with a Se-S interaction in the redox-regulated transcription factor NF-κB (41). Another transcription factor whose activation may be regulated by disulfide bond formation is the estrogen receptor (ER). ER binding to DNA is strongly influenced by the redox state of the cell (46), as the ER contains cysteines in zinc (Zn) fingers that are susceptible to oxidation and internal disulfide formation, which can prevent DNA binding (1). Dimerization of the ER is required for activity, with one Cys₄-type zinc finger found
in the DNA-binding domain and one in the dimerization domain (46). Whittal et al. (46) showed that ER redox sensitivity is likely related to the Zn finger in the dimerization domain and that Zn is expelled from the Zn finger previous to disulfide formation when treated with H₂O₂ or diamide. Jacob et al. (17) detailed the conditions under which Se compounds can catalyze the release of Zn from Zn fingers, even under reducing conditions, as found in vivo.

The effects of estrogen are mediated through the ER. The roles of estrogen in prostate cancer have recently been reviewed (15, 37) and animal models and human epidemiological studies have implicated estrogen as a promoter of prostate cancer in its early stages (40). If Se compounds displace Zn in the ER Zn fingers and catalyze disulfide bond formation, then ER binding to DNA and expression of ER-regulated genes should decrease. To test this hypothesis, we examined ER binding to its DNA response element and gene expression levels for estrogen-regulated genes in human prostate cancer cells (LNCaP) treated with control (50 nM) or high (5 µM) concentrations of Se.

MATERIALS AND METHODS

Cell Culture

LNCaP human prostate cancer cells (cell line established from a 50-year old male lymph node metastasis, ATCC, Manassas, VA) were maintained in RPMI 1640 with phenol red (ATCC, Manassas, VA) modified by ATCC to contain: 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, and 150 mg/L sodium bicarbonate. Media was further supplemented to contain 10% Fetal Bovine Serum (FBS, HyClone, Logan, UT), 1% penicillin-streptomycin solution (Sigma-Aldrich Corp., St.
Louis, MO), and 30 µM (±)-α-tocopherol (Sigma-Aldrich Corp., St. Louis, MO). Cells were incubated at 37°C in 5% CO₂.

Conventional cell culture media typically provide concentrations of antioxidants far lower than those found in human plasma. Liest et al. (27) showed that this may increase the risk of peroxide-induced genotoxicity. In their trials, supplementation with 50 nM Se and 30 µM α-tocopherol eliminated single strand DNA breaks induced by H₂O₂ and maximized the activity of Se-dependant glutathione peroxidase in all cell lines examined. In our experiments, all media were supplemented to provide these concentrations of antioxidants.

To examine the effects of Se treatment, 90% confluent 25 cm² flasks of LNCaP cells were passaged 1:2 to new 25 cm² flasks and equilibrated for 48 hours in phenol red-free RPMI 1640 medium (Sigma-Aldrich Corp., St. Louis, MO) modified to be identical to maintenance medium above, except that estrogen content was supplemented to 30 pg/mL to be within the range of normal male serum levels (22). Prostate cancer patients do not have significantly increased or decreased levels of estrogen compared to healthy men (32, 42). Phenol red-free medium was used to eliminate any possible estrogenic effects of phenol red (2). After 48 hours, medium was completely removed and fresh phenol red-free estrogen-supplemented medium was added. Half of the flasks received medium containing 50 nM Se and half received medium providing 5 µM Se as methylseleninic acid (CH₃SeO₂H, MSA). Cells were then incubated 72 hours and harvested.
Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from harvested cells using a Panomics Nuclear Extraction Kit (Panomics, Inc., Redwood City, CA), with the following modifications: As cells were cultured in flasks, rather than dishes, cells were trypsinized with 0.025% Trypsin/0.265 mM EDTA solution (Cambrex Bio Science Walkersville, Inc., Walkersville, MD), removed from the flasks and washed twice with phosphate buffered saline (PBS) (after 10 minute spins at 125 x g following each wash). Before proceeding with the isolation as outlined in the Panomics kit instructions, cells were then transferred to 1.5 mL Eppendorf tubes in Buffer A mix and the remainder of the protocol was followed as outlined, including dialysis with Buffer C and Slide-A-Lyzer Dialysis cassettes (Pierce, Rockford, IL). Protein was quantified using Bradford reagent (Sigma-Aldrich Corp., St. Louis, MO).

Table 1 lists the forward and reverse sequences of the 46-mer estrogen response element (ERE) from the Xenopus vitellogenin A2 gene used in previous studies of ER interaction (36), which was chosen for use in these experiments. Single strands (Bio-Synthesis Incorporated, Lewisville, TX) were mixed to a 5 µM concentration each, heated to 75°C for 15 min, then allowed to cool slowly at room temperature (RT) for 30 minutes and stored at -20°C. Aliquots were end-labeled with P$^{32}$ using T4 polynucleotide kinase (Fisher BioReagents, Fairlawn, NJ). Incubation followed the formula of García Pedrero et al. (10) as follows: 10 µL buffer B (20 mM HEPES-KOH, pH 7.9; 10 mM MgCl$_2$; 1 mM EDTA; 10 % (vol/vol) glycerol; 100 mM KCl; 0.2 mM phenylmethylsulfonyl fluoride (PMSF); 0.2 mM dithiothreitol; 0.5% Nonidet P-40; and protease inhibitors), 1 µL Poly (dIdC)*Poly(dIdC) (prepared to 1 µg/µL, Amersham
Biosciences, Piscataway, NJ), 1 µL 150x cold competitor, when appropriate (5 µM ERE
diluted 1:2), 5 µg nuclear protein, and water to equal 20 µL, then incubated 15 minutes at
4°C. Next, 1 µL radiolabeled probe was then added, reaction mixtures were incubated 1
hour at 4°C and 30 minutes at RT, and loaded into the lanes of a 5% polyacrylamide gel
(polyacrylamide:bisacrylamide, 39:1) in 0.5x TBE. The gel was pre-run (before addition
of reaction mixtures) at 150 V until mA reached ~18 (1-1.5 hrs). Samples were then
loaded and run at 100 V for 2-3 hrs (until dye front (bromophenol blue/xylenol orange)
was 2-3 cm from the bottom). Gels were dried under vacuum at 80°C for 60 min,
exposed to Kodak BioMax MS film (Eastman Kodak Company, Rochester, NY) at -80°C
for 1-18 hrs (depending on the age of the probe), and developed. Densitometric analysis
(Spot Densitometry, AlphaEase software, Alpha Innotech, San Leandro, CA) was
performed on multiple exposures of three separate EMSA gels.

Gene Selection

The genes chosen for study in the run-on transcription assay and steady-state
mRNA experiments are regulated by estrogen in the prostate, and are expressed in the
LNCaP cell line. IGFBP10 (14) is important for growth and proliferation, and is one of a
family of insulin-like growth factor binding proteins. In previous work high Se intake
induced a significant reduction in IGF-1 levels in circulation in rats (12), suggesting Se
could affect IGFBP10 levels. Cdk2 (21, 28), a regulator of the cell cycle, is involved in
prostate tumor progression and is associated with uncontrolled tumor cell proliferation. It
is more highly expressed in LNCaP cells than the prostate cancer cell lines PC3 or
DU145. Cathepsin D (24, 25) is a lysosomal protease. It plays a role in cell proliferation
and development in prostate cancer, and is also highly expressed in LNCaP cells. HpS2
(18, 26), also known as trefoil factor 1 (TFF1), serves as a marker of malignancy in various cancer types, including prostate (5). It is expressed in LNCaP cells, and is induced by estrogen.

**Run-on Transcription Assay**

To determine the effects of Se on transcription rates for each gene of interest, nuclei were isolated according to instructions in the Panomics Nuclear Extraction Kit, stopping after gentle lysing of the cellular membrane but maintaining nuclei intact. Run-on transcription was carried out according to the method of Patrone et al. (34) using biotinylated UTP (Roche Diagnostics Corp., Indianapolis, IN) to label nascent transcripts and streptavidin-coated magnetic beads (Dynal Biotech LLC, Brown Deer, WI) to isolate them. Reverse transcription was performed using Superscript II RNase H− Reverse Transcriptase (Invitrogen Life Technologies Corp., Carlsbad, CA) and first strand cDNA was purified using the QIAquick PCR Purification Kit (QIAGEN Sciences, Inc., Germantown, MD). Nascent transcripts for the genes of interest were quantified using real-time RT-PCR.

Primers were designed for published sequences (GenBank) using OMIGA 2.0 software (Genetics Computer Group, Inc. Madison, WI) (Table 1). Optimum temperatures for primer annealing were determined using a RoboCycler (Stratagene, La Jolla, CA) with a variable temperature block. Gel electrophoresis of RoboCycler results confirmed amplification of a single band of the expected size. Transcript levels were then quantified using the Roche LightCycler (Roche Diagnostics Corp., Indianapolis, IN) and SYBR green I dye. The annealing temperature was chosen based on the strongest amplification band in the RoboCycler experiments. Analysis of raw data from the
LightCycler was standardized by using the Fit Points method, arithmetic baseline, with the noise band set just below the point at which the second derivative maximum method calculated highest amplification efficiency. The crossing point was then set at the second derivative maximum point. Two fit points were used, with the first point below the crossing point and the second above, for as many samples as possible. Transcription rates for 18S rRNA were also quantified and used for normalization. For each gene 6 Lightcycler runs were performed. Each run included 3 replicates for both 50 nM and 5 µM Se treatments.

**Steady State mRNA Levels**

To determine the effects of Se on steady state mRNA levels for each gene of interest, total RNA was isolated from LNCaP cells treated with 50 nM or 5 µM Se, using TRIzol reagent (Invitrogen Life Technologies Corp., Carlsbad, CA). Concentration was determined spectrophotometrically for each treatment and integrity was verified by agarose gel electrophoresis. Total RNA (5 µg) was reverse transcribed into cDNA and purified as described above. Primers and annealing temperatures were used as in the quantitation of nascent transcripts. Quantitation of steady state mRNAs by real time RT-PCR for each gene of interest was identical to that described above. Three runs of three replicates each of both 50 nM and 5 µM Se treatments were performed on the LightCycler, except for hpS2, for which a fourth run was completed.

**Statistical analysis**

For electrophoretic mobility shift assays, t tests were used to compare the relative mean band densities for control and high Se-treated samples.
Statistical analysis was performed using a randomization test with the concentrations calculated by the LightCycler software. These calculated concentrations were compared to those derived using the method of Pfaffl (35) and found to be nearly identical. To examine relative gene expression, the mean of the replicates of the gene of interest was normalized by dividing by the mean of the replicates for 18S rRNA. For each gene the normalized value for the high Se group was divided by the normalized value for the adequate Se group to give a ratio for relative gene expression.

To determine statistical significance, all replicates of calculated concentrations for the gene of interest in control Se-treated cells were randomized together with all replicates for 18S rRNA in the same cells. The same randomization was done with all replicates for the gene of interest and for 18S in high Se-treated cells. Following this randomization the same calculations as described above were performed to derive a final ratio expressing relative gene expression. This randomization and recalculation was repeated 1000 times, and the number of recalculated ratios greater than and less than the original calculated ratio were tallied to determine the probability of obtaining the original ratio simply by chance. The null hypothesis - that the treatment had no effect on gene expression - would predict an original ratio of 1.0. The only assumption made about the data was that of independence, as each sample was run in a separate capillary. In this randomization model no assumptions are made about the distribution of the original calculated concentrations or the values calculated from the randomized data. A more detailed description of these statistical methods is in preparation.
RESULTS

Figure 1 shows typical results from an electrophoretic mobility shift assay. Densitometric analysis was performed on multiple exposures of three separate EMSA gels. These analyses showed that the band in the control Se lane accounted for 53.8 ± 6.9 % (mean ± SD) of the total density of the control and high Se bands combined, while the high Se band accounted for 46.2 ± 6.9% of the total. This difference was not statistically significant. Disappearance of the band resulting from the addition of unlabeled oligonucleotide probe to the incubation confirmed the specificity of binding to this probe.

Figure 2 shows the results of the run-on transcription assays. The ratios of high:control, adjusted for 18S, for IGFBP10, Cdk2, and Cathepsin D are 0.994, 0.880, and 0.894, respectively. The probability that the ratio of transcription rates (high:control) was due strictly to random chance for IGFBP10 was 0.385; for Cdk2, 0.347; and for Cathepsin D, 0.372.

Results from the analysis of steady state mRNA levels for each gene are shown in Figure 3. The ratios of high:control for IGFBP10, Cdk2, Cathepsin D, and hpS2 are 1.231, 1.108, 1.029, and 1.126, respectively. The probability that the 23% difference in steady state levels of mRNA between high and control Se samples for IGFBP10 occurred by random chance was 0.034. For Cdk2, the probability was 0.119; for Cathepsin D, 0.413; for hpS2, 0.196.
DISCUSSION

Selenium metabolites, such as MSA, are critical for the chemopreventive effects of Se (6). Ip et al. showed that monomethylated forms of Se cleaved from larger forms, such as selenocysteine or Se-methylselenocysteine, provide the chemopreventive effects (16). Cultured cells have low activity of the β-lyase necessary to cleave monomethylated metabolites from dietary forms of Se, so a monomethylated form must be provided directly (19). A concentration of 5 µM Se is commonly used in cell culture studies (20, 43, 49), and is between maximum serum achievable levels of Se and average population values (33). Serum levels of 5 µM can be obtained from a high Se diet or supplementation.

Ganther has proposed that one mechanism by which Se may reduce cancer risk is by covalent structural modification of cellular proteins, including transcription factors (8, 9). Others have demonstrated interactions of Se with reactive sulfhydryls in proteins (11), and we have recently provided evidence consistent with such interaction in the redox-regulated transcription factor NF-kB (41). However, our results in cultured cells do not suggest as dramatic effects for Se on Zn fingers as those shown by Jacob et al. in vitro (17). Although their experiments were performed in a reducing environment to match the state of the cell, Jacob et al. tested phenyl derivatives of Se, which are more stable than their aliphatic counterparts, but are not as biologically relevant.

Zinc finger proteins constitute as much as 1% of all human gene products (1) and MSA strongly reacts with zinc-sulfur clusters of metallothionein (MT) (17). Quantitatively, the concentration of ER is minor compared to other Zn finger-containing proteins that may react with Se. LNCaP cells express ER-beta at a low level, and express
little or no ER-alpha (26, 30). It is possible that the MSA used in our experiments
interacted primarily with other, more abundant zinc fingers present in the cell,
diminishing interaction with the ER.

We did find some evidence of interaction between Se and the ER (see Figure 1),
but in this cell line under the conditions used it was minor, or its effects were negated by
other factors. For example, ER binding to DNA requires a number of co-factors. If
everything else is in place, a modest effect of Se on ER Zn fingers may not have been
sufficient to significantly alter DNA binding.

The large number of proteins required for gene transcription in vivo could further
explain why significant down-regulation of estrogen-regulated gene products did not
occur. Such proteins were not present in the experiments of Jacob et al. (17) or the
examination of the ER Zn fingers by Whittal et al. (46). Once such proteins are in place, a
small effect on ER binding may not have been sufficient to significantly down-regulate
gene transcription. Furthermore, although the genes examined are known to be estrogen
regulated, other factors or pathways could also activate their transcription, independent of
estrogen.

Though one significant value resulted from the steady state mRNA assay, there is
uncertainty that a 23 % higher level is biologically significant. As the other genes
examined also resulted in slight, though non-significant, increases, there was likely no
overall biological effect on steady state mRNA levels of the estrogen-regulated genes
examined.

Other studies provide evidence that Se reacts with sulfhydryl groups (17).
However, that reaction, if it occurs in the ER, has a minimal effect on the binding of ER
to DNA and its regulation of gene expression. Our results are internally consistent.

Without a significant change in DNA binding a significant change in expression of ER-regulated genes would not be expected. Our findings do not refute the hypothesis that Se can alter transcription factor structure and binding to DNA, and therefore regulation of gene expression. They simply do not provide support for the hypothesis that this mechanism is operative in the case of the ER in LNCaP prostate cancer cells.

Perspective: Interaction of Se with sulphydryl groups in proteins can have significant biological effects. However, in LNCaP cells, the effects of high Se treatment on the ER and ER-regulated gene expression appear to be minimal. Other transcription factors whose binding to DNA is dependent on relatively few co-factors, and genes whose expression is regulated by those transcription factors alone, would be more likely to be affected by a change in one of the participating factors, such as Se complexing with reactive sulphydryl groups. Identifying Zn fingers in such transcription factors or co-factors would allow analysis of Se’s effects while minimizing confounding factors in vivo. LNCaP cells express ER-beta at a low level, and express little or no ER-alpha (26, 30). Other cell types or cell lines with higher expression of ER might reveal a more significant effect of Se. Alternatively, other proteins of high abundance, which are known to react with Se, are more likely to be affected by a change in Se status or availability (17).

**Abbreviations:** EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; ERE, estrogen response element; MT, metallothionein; MSA, methylseleninic acid.
REFERENCES


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<td>ERE reverse</td>
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<tr>
<td>pS2 reverse</td>
<td>5'-AGC CCT TAT TTG CAC ACT GG-3'</td>
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FIG 1. Electrophoretic Mobility Shift Assay (EMSA). Lane 1: Probe only. Lane 2: 50 nM Se. Lane 3: 5 µM Se. Lane 4: 50 nM Se + 150x competitor. Lane 5: 5 µM Se + 150x competitor. Arrow indicates ER/ERE band. The density of the band in Lane 3 was a non-significant 16% lower than the density of the band in Lane 2, based on three separately run gels with multiple film exposure times for each.
FIG 2. Run-on transcription assay: Ratio of the mean of the 5 µM Se-treated replicates divided by the 50 nM Se-treated replicates by gene, each adjusted for 18S rRNA. Bars represent the ratio of the means of LightCycler calculated concentrations for all replicates as: high (gene)/ high (18S) divided by control (gene)/control (18S). The probability that the ratio was due strictly to random chance for IGFBP10 was 0.385; for Cdk2, 0.347; and for Cathepsin D, 0.372. Error bars are not possible using this statistical model. See text for further discussion.
FIG. 3. **Steady-state mRNA: Ratio of the mean of 5 µM Se-treated replicates divided by 50 nM Se-treated replicates by gene, each adjusted for 18S rRNA.** Bars represent the ratio of the means of LightCycler calculated concentrations for all replicates as: high (gene)/ high (18S) divided by control (gene)/control (18S). The probability that the 23% increase in steady state levels of mRNA for IGFBP10 occurred by random chance was 0.034. For Cdk2, the probability was 0.119; for Cathepsin D, 0.413; for hpS2, 0.196. * indicates probability <0.05. Error bars are not possible using this statistical model. See text for further discussion.
Selenium

Selenium (Se), found directly beneath sulfur on the periodic table, is incorporated into the food chain through plants that incorporate it into cysteine and methionine in the place of sulfur (11). Se was initially recognized for its toxic effects on livestock consuming plants of the genus *Astragalus*, *Xylorrhiza*, *Oonopsis*, and *Stanleya* in the western part of the United States in 1933 (75). It was recognized as an essential element for laboratory rats in 1957 (85), followed by sheep and chicken. The requirement of Se for glutathione peroxidase (GPx) activity in mammals followed in 1973 (81, 91). Se plays an important role in enzymatic reactions as part of the amino acid selenocysteine (SeCys). However, Se appears to have anti-tumorigenic activity at levels far exceeding Se intakes that maximize expression of known SeCys-containing enzymes (46, 72). Part of the cancer protection seen through the action of Se is derived from Se-dependent GPx's and thioredoxin reductase (TR) (82). But again, additional benefits are seen at higher intake levels, suggesting Se supplementation may affect cell cycle regulation, apoptosis, immune function (57), or carcinogen metabolism (12, 72, 79). Based on the understanding that Se is beneficial above levels required to maximize the above enzymes, Se supplementation may be beneficial even in those already consuming adequate levels of Se in the diet (46, 72). Selenium metabolites, such as methylseleninic acid (CH$_3$SeO$_2$H, MSA), are critical for the chemopreventive effects of Se (21).
Dietary Selenium

Dietary Se is consumed as SeCys from animal origin and as selenomethionine (SeMet) from plant sources (45). Other forms include selenized yeast, selenohomocysteine, selenocystathionine and Se-adenosylselenomethionine. Selenium supplementation should begin early in life, and continue for the entire lifespan for optimal cancer protection (50). Methylation of Se compounds by the body produces less toxic forms and is the principal means of excreting excess Se (30).

Selenium metabolism

Selenite (H₂SeO₃) supplementation results in the formation of selenodiglutathione (GSSeSG). Reduction of GSSeSG by thiols and NADPH-dependent reductases, including glutathione reductase, releases hydrogen selenide (H₂Se) via glutathione selenopersulfide (GSSeH, 6). It can also be formed through the action of β-lyase on selenocysteine. Hydrogen selenide is then converted to selenophosphate (H₃SePO₃), making Se available for synthesis of body proteins (103). Selenomethionine is also incorporated non-specifically for methionine and is the major form of Se in selenized yeast, used in chemopreventive studies (98). Se-methylselenocysteine, found in selenized garlic and other Allium vegetables, is also chemopreventive, with several advantages over selenomethionine (98). This is likely due to the fact that it is a precursor to MSA.

Different chemical forms of Se have different biological effects. MSA, a monomethylated low molecular weight Se compound, is a common metabolite of many anticarcinogenic forms of Se (30). Se-methylselenocysteine, and other large organic naturally occurring anticarcinogenic food forms of Se, must be broken down to a monomethylated form by a β-lyase. However, cultured cells have low activity of the β-
lyase necessary, so a monomethylated form must be provided directly (49). Selenite, the standard form of Se used for decades in animal feeding experiments, comprises only a minuscule percentage of total food Se and its metabolism in the gut and liver makes it extremely unlikely that cells would be exposed in vivo to this chemical form.

**Selenoproteins**

The principle form of Se found in mammalian proteins is SeCys (93). It is incorporated during translation utilizing a UGA codon in the mRNA, normally a stop codon, with the aid of a hairpin secondary structure in the 3'-untranslated region of the mRNA (5). In total, 21 selenoproteins have been identified. These include four GPx, two deiodinases, two thioredoxin reductases and selenophosphate synthetase (1). Other selenoproteins, such as selenoproteins P and W, are not clearly defined (46).

The first mammalian selenoprotein identified was cytosolic glutathione peroxidase (GPx1). Not long after, glycosylated plasma glutathione peroxidase (GPx2), gastrointestinal glutathione peroxidase (GPx3) and phospholipids-hydroperoxide glutathione peroxidase (GPx4) were discovered. GPx1,2,3 are tetrameric, with one selenocysteine found on each subunit. GPx4 is a monomer. GPx enzymes reduce hydroperoxides, such as H₂O₂, fatty acid hydroperoxides, cumene hydroperoxide, cholesterol hydroperoxides and t-butyl hydroperoxide (1). After GPx1, type 1 iodothyronine deiodinase (5'DI) was next discovered (72, 93). This enzyme is a homodimer with one selenocysteine at each of two active sites. 5'DI catalyzes the 5'-deiodination of L-thyroxine to tri-iodo-L-thyronine (93). Thioredoxin reductase (TR) is a Se-dependent homodimeric flavoprotein that reduces thioredoxin in an NADPH-dependent manner (1, 5). It can also reduce 5,5'dithiobis-2-nitrobenzoic acid (DTNB) and
insulin (96). The thioredoxin/TR system reduces ribonucleotides to deoxyribonucleotides, as well as maintaining redox balance in the cell and regulating the activity of transcription factors. Selenoprotein P is a Se-containing glycoprotein, providing about a third of the total selenium in plasma (10). It contains up to 10 SeCys residues and may have metal binding activity (46), or be useful as a marker for Se status (28). Selenoprotein W is localized to brain tissue and does not correlate to normal Se distribution (20).

**Se and cancer**

Cancer mortality rates for major cancer sites are significantly higher in low selenium countries (16) and dietary intake of Se correlates inversely with total age-adjusted cancer mortality in 27 countries (7). For hepatocellular carcinoma (HCC), the group receiving 200 µg of Se yeast showed no cases of HCC, while the placebo group had seven individuals develop HCC. The study was carried out in 226 hepatitis B antigen carriers (115).

Se is independently predictive of cancer mortality in a sample of Belgian adult males, but not females (62). The serum Se levels were already quite high within the population. High serum Se levels have been associated with reduced risk of prostate cancer among blacks and whites, especially if serum α-tocopherol levels were low (106). In breast cancer, low serum selenium levels appear to be the result, not the cause, of breast cancer (68). A meta-analysis found Se to be protective against lung cancer in areas where average Se levels are low (117). For women with ovarian cancer undergoing chemotherapy, Se supplementation increased GPx levels and decreased negative symptoms associated with the therapy (88). An inverse association between toenail Se
and colon cancer has also been found, though in the same study, no significant correlation was found for breast or prostate cancer (32). Significant inverse associations have also been found between baseline serum Se and death from esophageal squamous cell carcinoma and gastric cardia cancer (109).

**Mechanisms of cancer prevention by Se**

The mechanisms for precisely how Se exerts its protective effects have not been fully explored, though mounting evidence suggests many possible molecular targets (56). Micromolar levels of monomethylated forms of Se cause apoptosis in p53 null phenotypes, independent of DNA damage (34). Se also inhibits cyclin dependent kinase 2 (cdk2) and protein kinase C. Ganther (30) proposed Se, particularly monomethylated forms such as methyl selenol (CH$_3$SeH) and/or MSA, could form Se adducts like selenotrisulfide (S-Se-S) or selenenylsulfide (S-Se). This could affect cysteine/disulfide transformations and affect a number of redox regulated proteins, particularly transcription factors (36, 80). Though its function in *in vivo* chemoprevention is not well established, the thioredoxin/TR system is upregulated with Se supplementation in the nutritional to supranutritional range, but is inhibited if Se causes growth inhibition, presumably by its actions external to TR. Se shows antimutagenic activity, preventing malignant transformation of normal cells and activation of oncogenes (50). These effects are likely due to maximized activity of GPx enzymes, which protect cellular components, including DNA from oxygen radical damage. Selenoenzymes also have established roles in carcinogen metabolism, immune system function and oxygen metabolism (50). Methylated forms of Se also appear to have significant effects on carcinogenesis, and are required at much lower doses for similar effects than inorganic selenite (31, 89, 93, 112).
Se-methylselenocysteine or Se-methylselenocysteine selenoxide are cleaved by a β-lyase to methyl selenol or MSA (2). MSA increases poly (ADP-ribose) polymerase cleavage and upregulates caspases 1, 3, 6, 7, 8, 10, and 12 toward increased apoptosis in PC-3 human prostate cancer cells. The effect was significantly enhanced when vitamin E succinate (D-α-tocopheryl succinate) was also added (118). The genes GADD153, CHK2, p21\textsuperscript{WAF1}, cyclin A, CDK1 and DHFR may be targets of MSA in its role of impeding cell cycle progression (24). Se may also affect inhibition of cell invasion, DNA repair and stimulation of transforming growth factor beta signaling (24).

Our laboratory has identified many genes whose expression is differentially regulated in rats by dietary Se intake (15, 52, 76, 113). These findings have suggested possible molecular mechanisms for the reported chemopreventive effects of Se against prostate cancer. Among the genes our lab has shown to be Se-regulated are those for the estrogen receptors (ERs) (39) and the enzyme estrogen sulfotransferase (EST) (113). Focusing on Se’s protective effects against prostate cancer, our more recent experiments in rats showed that dietary Se intake affects blood estradiol concentrations, liver EST activity, and expression of estrogen receptor beta (ER\textbeta) in the dorsal prostate (39). Furthermore, the roles of estrogen in prostate cancer have been reviewed (9, 37). Both animal models and human epidemiological studies have implicated estrogen as a promoter of prostate cancer (95).

Ganther (30) proposed that one molecular mechanism by which high Se concentrations may affect cancer risk is by catalyzing disulfide bond formation in cellular proteins, including transcription factors. Our lab has provided evidence to support this hypothesis by showing that high Se treatment of LNCaP cells reduces binding to DNA of
the redox-regulated transcription factor NF-κB, and decreases expression of NF-κB-regulated genes. Like NF-κB, ER binding to DNA is strongly influenced by the redox state of the cell (110). The ER DNA-binding domain contains cysteines in zinc fingers that are susceptible to oxidation and internal disulfide formation, which prevents DNA binding. As in NF-κB, high concentrations of low molecular weight Se compounds may catalyze disulfide bond formation in ER, reducing its binding to DNA and decreasing expression of ER-regulated genes (29).

Se adduct formation in cysteine residues of proteins inactivates essential thiol groups of transcription factors and may be a mechanism for the toxic effects of Se (55). In the presence of protein disulfide isomerase, the addition of selenite (1 µM) resulted in rapid formation of disulfide bonds in ribonuclease, aiding folding and restoring its activity in a reducing environment (70). Zinc (Zn) is often complexed between multiple cysteine residues. The high affinity for Zn of methyl seleno-compounds could remove the Zn, resulting in disulfide bridges, activating or inactivating enzymes (36). Proteins shown to be modified by redox mechanisms such as this include protein kinase C (36), and the transcription factors AP-1 and NF-kB (55, 92). In one case, cells were depleted of Se, then supplemented from 10-100 nM, with suppression of NF-kB as the dose increased (71).

Redox control of zinc finger proteins has been recently reviewed by Baldwin and Benz (4). Dimerization of the ER is required for activity and two Cys4-type zinc fingers can be found in the DNA-binding domain. Because of these zinc fingers, the ER is expected to be redox sensitive. Jacob et al. (47) detailed the conditions under which Se compounds can catalyze the release of zinc from zinc-sulfur coordination sites. Possible
targets include metallothionein and the ER. Chen and Maret (14) provide additional evidence of the reactivity of Se compounds in zinc/thiolate coordination sites in metallothionein. The release of zinc from zinc-sulfur clusters may be an important chemopreventive mechanism. Other reviewers lend support to the important role of zinc finger oxidation, focusing on transcription factor proteins (111) and physiological consequences (108). When zinc is coordinated in the zinc finger, the Cys residues are more resistant to oxidation, further supporting the unique role of Se compounds to carry out the oxidation (108). Physiologically, long-term oxidation of zinc fingers, and hence inactivity, of Sp1 and ER may be the result of lost cellular homeostasis between reactive oxygen and quenching systems, altering the normally reducing environment of the cell and explaining certain aspects of aging and ER negative cancers (14).

Prostate cancer

Prostate cancer is the most frequently diagnosed cancer in American men and the second leading cause of cancer deaths. The American Cancer Society estimates that prostate cancer will account for 33% of new cancers in men, totaling over 230,000 new cases, with 29,900 deaths in 2004 (94). Generally arising from prostatic epithelia, prostate cancer begins androgen responsive, and progresses to an androgen independent stage. Risk increases with age, obesity, animal fat consumption, red meat consumption, and among African-Americans, while cereals, vegetables, and vitamin D appear to be protective (25). However, the European Prospective Investigation into Cancer (EPIC) study found no correlation between fruits and vegetables and prostate cancer (53). Giovannucci et al. (33), who found no protective effect for cruciferous vegetables in older men, did provide some evidence that cruciferous vegetables may be protective early
in prostate cancinoogenesis. Dairy products and calcium are associated with a greater risk of prostate cancer (13), and androgen levels and reactive oxygen species are also linked to increased levels (17). On the other hand, moderate to high consumption of fatty fish is associated with decreased risk (23, 97), and lifetime occupational physical activity may have a beneficial effect on the occurrence of prostate cancer (3). A traditional Japanese diet, typically rich in soy-based products and fish, may be protective (90).

**Prostate cancer diagnosis**

Various methods are utilized in the diagnosis of prostate cancer (83). The earliest method of identifying asymptomatic prostate cancer was through digital rectal examination. The test for prostate specific antigen (PSA) in the blood allowed for a quantitative measurement of prognosis, with higher levels indicating higher levels of prostate cancer growth or activity (105). Transrectal ultrasonography (TRUS) produces an image of the prostate using sound waves. TRUS is used in conjunction with prostate fine needle biopsy to obtain a tissue sample for testing. Fine needle biopsy is also utilized to test for metastasis in pelvic lymph nodes. A PSA test combined with TRUS and prostate needle biopsy is the best method for an accurate diagnosis (83). A complete blood count (CBC) looks for abnormal blood cells to determine if the cancer has spread to the bone marrow (7). A computed tomography (CT) scan can also be used for prostate cancer diagnosis, but is not as accurate as TRUS (42).

**Prostate cancer treatment**

Treatment options include radiation therapy, hormone therapy, chemotherapy, or radical prostatectomy (22). Radiation therapy targets the prostate and surrounding tissue with ionizing radiation in an effort to destroy the cancer cells while minimizing normal
cell death. Hormone therapy generally targets testosterone or other androgens, via antiandrogens or castration to abolish hormonal stimulation, which may contribute to cancer cell growth. Chemotherapy utilizes various drugs given systemically which interrupt aspects of cell growth, division, or signaling in an attempt to destroy the cancer cells without killing the host. Treatments are completed in cycles to allow recovery. Chemotherapy drugs are becoming more specific to cancer, and less toxic to the host. Radical prostatectomy is the removal of the prostate, effectively eliminating the cancer, assuming no metastasis had occurred and the operation was successful at removing all transformed cells. Side effects often include incontinence and/or impotence.

**LNCaP prostate cancer cell line**

The Lymph Node Cancer of the Prostate (LNCaP) cell line was originally established (43), and later described (44). LNCaP cells grow slowly, generally in a monolayer, though with some clumping, and only attach loosely to the substrate. Both the androgen and estrogen receptors were found to be present. Prostatic acid phosphatase and prostate specific antigen (PSA) levels were both higher than normal prostate tissue. Conventional cell culture media typically provide concentrations of antioxidants far lower than those found in human plasma. This may increase the risk of peroxide-induced genotoxicity (64). To eliminate this possibility we supplement all cell culture medium with 50 nM Se and 30 µM α-tocopherol. These concentrations eliminated single strand DNA breaks induced by H₂O₂ and maximized the activity of Se-dependant glutathione peroxidase (GPX, an antioxidant) in the cell lines examined by Leist et al. (64).
**Prostate cancer and Se**

Because of its high incidence and long latency period prostate cancer is an ideal candidate for chemoprevention by dietary means. The Nutritional Prevention of Cancer Trial showed a marked reduction in prostate cancer incidence in men receiving a daily supplement of 200 µg selenium (Se) as selenized yeast (17, 18, 20). A protective effect for Se has been confirmed by other reports that dietary Se intake (84), prediagnostic serum Se concentration (41, 77, 100), and toenail Se (114) are inversely correlated with prostate cancer risk.

The thoredoxin/TR redox system, important in cell growth and apoptosis in prostate cancer cells, requires Se (27, 78). Low serum levels of Se are associated with a higher risk for prostate cancer (16, 84, 87). Supplemental Se appears to reduce the incidence (8, 116), and higher levels of Se may even slow the progression of prostate cancer tumors (66). Many mechanisms have been proposed for Se action in prostate cancer. These include: oxidative protection (GPx and others), cytotoxic effects, carcinogen metabolism modulation, apoptosis, angiogenesis inhibition, TR action and cell cycle regulation (49, 58). Venkateswaran et al. (102) examined four prostate cancer cells lines, and found Se treatment caused G1 arrest and an 80% reduction of S phase in LNCaP cells. Se treatment induced the cyclin-dependent kinase inhibitors Cip1/p21 and Kip1/p27. In each case, androgen dependence was necessary for the effects (102).

**Estrogen, Estrogen receptors and the Estrogen Response Element**

Estrogen plays an important role in male physiology, affecting brain function, cardiovascular physiology, bone maturation and resorption, lipid and skin metabolism, sex interest, and in the aging male, prostate (7, 104). Derived principally from the
androgen testosterone, estrogens are formed through peripheral aromatization in fatty tissue (35). Estrogen levels do not decline with age in men (54), and prostate cancer patients do not have significantly increased or decreased levels of estrogen compared to normal men (73, 101). However, sex hormone binding globulin increases, decreasing available estrogen (7, 54). Androgens decrease with age, suggesting the changed androgen/estrogen ratio may be important, particularly in prostate (7, 54).

The estrogen receptor alpha (ER\textsubscript{α}) (99) induces transcription of target genes by binding to promoters of responsive genes. It is a member of a superfamily of transcription factors that bind to \textit{cis}-acting enhancer elements in the promoter region (26). ER\textsubscript{β} is highly homologous to ER\textsubscript{α} in DNA and hormone binding domains, sharing 96 and 58% homology, respectively (74). In human males, expression is highest in the testis (74). Both bind 17-β-estradiol with similar affinities. LNCaP cells express ER\textsubscript{β}, though at a low level (67). This expression level is higher than that found in hormone-refractory cell lines.

Both ER\textsubscript{α} and ER\textsubscript{β} bind to the consensus vitellogenin A2 estrogen response element (ERE) sequence GGTCAnnnTGACC (59). However, there are many genes which respond to ERs that differ from the A2 ERE by one or more base pairs (69). ER\textsubscript{α} more potently activates promoters containing perfect or imperfect EREs, though ER\textsubscript{α} and ER\textsubscript{β} have similar affinities for them. The individual ERE differences may account for the different transactivating effects (69).

\textbf{Estrogen regulated genes}

Several genes are known to be regulated by estrogen in prostate, including expression in the LNCaP cell line. The genes described below meet both criteria. Cdk2
(51, 65) is involved in prostate tumor progression and is associated with uncontrolled
tumor cell proliferation, as it is a regulator of the cell cycle. It is more highly expressed in
LNCaP cells than the prostate cancer cell lines PC3 or DU145. Cathepsin D (60, 61) is a
lysosomal protease. It plays a role in cell proliferation and development in prostate
cancer, and is also highly expressed in LNCaP cells. PDL1 (40), a phosphatase important
for signal transduction, removes phosphate groups on serine or threonine residues from a
wide variety of phosphoproteins. HER2 (107), implicated in oncogenesis, is highly
expressed in LNCaP cells. Galectin 1 (40) binds to β-galactosidase in a calcium-
independent manner, is highly expressed in LNCaP cells, and plays a role in proliferation,
differentiation, and apoptosis. IGFBP10 (40) is important for growth and proliferation,
one of a family of insulin-like growth factor binding proteins. Se induced a significant
reduction in IGF-1 levels in circulation in rats (38), so thus may affect IGFBP10 levels.
Ha-Ras (60, 86) produces a monomeric GTPase signal transduction protein which is also
an oncogene. hPR (60, 82) encodes the progesterone receptor and is a key mediator of
proliferation and growth in the prostate. HpS2 (48, 63), also known as trefoil factor 1
(TFF1), serves as a marker of malignancy in various cancer types, including prostate
(19). It is expressed in LNCaP cells, and induced by estrogen.
REFERENCES--LITERATURE REVIEW


APPENDIX B

MATERIALS AND METHODS

A list of solutions and buffers, as well as an alphabetic list (by manufacturer) of purchased reagents can be found at the end of this appendix.

CELL CULTURE

LNCaP cells were maintained in RPMI 1640 with phenol red modified by ATCC to contain: 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, and 150 mg/L sodium bicarbonate.

For treatment, control and high Se flasks were cultured in phenol red free media otherwise identical to the above, except:
Estrogen levels were brought up to 30 pg/mL.
FBS contained 50 nM Se upon arrival, so automatically provided control levels.
For high Se treatment, methylseleninic acid (MSA) was added for a final concentration of 5 µM.

Cells were placed in the control treatment media for 48 hrs, then the media was completely changed (5 mL replaced per flask) in control and high flasks, with the high flasks receiving the extra MSA, for an additional 72 hrs. Cells were then harvested.

Media Formulation

RPMI 1640 (for LNCaP)-phenol red free - 500 mL

1. Put 50 ml of FBS (Fetal Bovine Serum) into 37°C water bath until thawed (takes approximately 1 hour).
2. Put 445 ml of deionized water into a 1 L beaker and stir.
3. Add ½ bottle powdered RPMI 1640 without phenol red and without NaHCO₃ (sodium bicarbonate) to beaker. Put a little deionized water into the media bottle, shake, and add this to the beaker. Mix Thoroughly.
4. Add thawed FBS to beaker.
5. Add 0.75 g NaHCO₃
6. Add 1.25 g glucose
7. Add 1.19 g HEPES
8. Add 0.055 g sodium pyruvate
9. Add 5 mL Sigma penicillin-streptomycin antibiotic solution
10. Add 500 µL 0.03 M α-tocopherol
11. Add 14.62 µL estrogen (1 µL/mL stock solution) for 30 pg/mL final []
12. Mix thoroughly
13. Check pH. Use 1N NaOH or 1N HCl to adjust pH to 7.2-7.3
14. Use sterile vacuum filters to filter media into sterile media bottles.
14. Flame and cap media bottles. Spray with ethanol, let air dry, and then parafilm.
15. Label each bottle with RPMI 1640 + FBS (Ph R free), initial and date.

**RPMI 1640 (for LNCaP)-with phenol red - 500 mL**

1. Put 50 ml of FBS (Fetal Bovine Serum) into 37°C water bath until thawed (takes approximately 1 hour).
2. Remove 55 mL media from ATCC LNCaP growth media (pre-prepared).
3. Add thawed FBS to media bottle.
4. Add 5 mL Sigma penicillin-streptomycin antibiotic solution
5. Add 500 µL of 0.03 M α-tocopherol
6. Use sterile vacuum filters to filter media into sterile media bottles.
7. Flame and cap media bottles. Spray with ethanol, let air dry, and then parafilm.
8. Label each bottle with RPMI 1640 + FBS (Ph R), initial and date.

**Maintenance conditions**

Cells were maintained at 37°C in an atmosphere of 5% carbon dioxide and 95% air. Media was changed every 2-3 days depending on growth and phenol red color change. The hood was sprayed with 70% EtOH and wiped down (with occasional cleaning with O-syl), and all instruments and equipment, including gloves, were doused and wiped with 70% EtOH to evaporation. The incubator was cleaned periodically with Roccal. Shelves can also be autoclaved. Media was filtered before each change using a 30 mL syringe and a 0.2 µm Acrodisc syringe filter and warmed in the incubator for at least 15 minutes. Generally all media was removed each time, though some growth benefit may be obtained by leaving one-third of the old media, replacing two-thirds. Aspirated floating cells in removed media were spun for 10 minutes at 125xg, then resuspended in 1-2 mL media per flask. Cells were passaged every one to two weeks, depending on need and growth. Excess cells were frozen in liquid nitrogen after a slow freeze wrapped in cotton in complete media plus 5% DMSO. Cells should optimally be cooled at 1°C per minute (fridge, then -20°C freezer, then -80°C freezer, then stored in liquid N₂).

After the initial receipt of cells from ATCC in a 1 mL aliquot, cells were grown in 25 cm² flasks in 5 mL media. Seven mL of media was added after the cells reached 50-60% confluence. Cells were passaged 1:2-1:4 depending on need, using 1 mL trypsin/EDTA solution per flask for a 3 min incubation followed by the addition of 2 mL media to inactivate the trypsin. Repeated pipetting with 10 mL pipet and electric pipetman to loosen cells from the flask and another 2 mL media rinse was followed by a 10 min spin at 125xg. In general, one flask for control and high Se treatment was required for nuclear protein and RNA isolation. Four flasks of each treatment were used for the run-on transcription assay.
Cell Passaging

**Materials:**
Complete RPMI 1640  
10 mL pipettes  
0.025% trypsin/ 0.265 mM EDTA solution  
culture flasks  
waste container

**Procedure:**

**Note:** Cells should be passaged at a minimum of 75-80% confluency.

1. Warm the medium and 0.025% trypsin/ 0.265 mM EDTA solution in incubator. (Trypsin is stored in 2 mL aliquots in 2 mL microcentrifuge tubes at -20°C.)
2. Observe cells and record observations.
3. Carefully aspirate medium and place in 15 or 50 mL centrifuge tube.
4. Add 1 mL trypsin to each 25 cm$^2$ flask. Make sure that the bottom of the flask is completely covered. Incubate in the incubator 3 minutes. **Note:** To avoid clumping, do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach.
5. Remove flasks from incubator. Add at least 2.0 mL of warmed, filtered (filter using a 30 mL syringe and a 0.2 µm Acrodisc syringe filter) complete medium (twice the trypsin). Rinse the side of the flask with the media until all the cells are removed. Repeat with another 2.0 mL media as a rinse.
6. Transfer trypsinized cells and media to the 15 or 50 mL centrifuge tube from step 3.
7. Centrifuge for 20 min. @ 1000 rpm. Discard supernatant. Add 1 mL fresh media to centrifuge tube per new flask and pipet with 10 mL pipet ~15 times. Add to new flasks, 1 mL each. Add 4 mL media to flasks for a total of 5 mL per 25 cm$^2$ flask.
8. **Subcultivation ratio:** 1:2-1:4. Pipet up and down several times so that cells are dispersed throughout the media.
9. Label flasks with cell type, subculture number, and date. Return to incubator.

**ELECTROPHORETIC MOBILITY SHIFT ASSAY (ref 4)**

**Nuclear protein isolation**

Nuclear extracts were isolated according to the Panomics Nuclear Extraction Kit with the following modifications:
1. Remove culture media from C and H 25 cm² flasks with 10 ml pipette and place in two labeled 15 ml tubes. Add 1 ml Clonetics 0.025% trypsin/0.265 mM EDTA to C and H flasks. Incubate in the incubator for 3 minutes.

2. Add at least 2 ml fresh media to neutralize trypsin. Pipette and wash repeatedly until all cell clumps are loose from the bottom of the flask. Add trypsin/cell/media mixture to above tubes. Add 2 ml more to rinse flasks, wash a few times, and add to original spin tubes. Spin 10 min at 125 g and pour off media into waste container.

3. Add 10 ml of PBS, pipette to mix, then spin 10 min. Repeat, pouring off PBS each time.

4. Refer to Panomics protocol starting with step two for cell culture, with the following modifications.

5. After preparing Buffer A mix, add 500 µL to 15 ml tubes, mix slightly, then pull pellet and add to labeled (C and H) 1.5 ml Eppendorf tubes. Add the second 500 µL of Buffer A mix, rinsing to get as many cells as possible from 15 mL tubes, and add to 1.5 ml Eppendorf. Pipette up and down several times. Continue with 150 rpm shake for 10 min in step 3 of the Panomics protocol. Use rotating shaker, placing Eppendorf tubes in ice in a 250 mL beaker.


7. In step 9, resuspension is difficult and required repeated vortexing, vertical sideways, etc. In one case, there was a large clump that would not mix even after repeated vortexing. It did not appear to interfere with the protein extraction, as sufficient yield was obtained.

8. I packed a 250 mL beaker with ice, laid the tubes on their sides on the ice in one of the metal holders in the rotating shaker next to the -20 C freezer.

9. The optional step 12 was also completed using the Slide-A-Lyzer dialysis cassettes and the accompanying protocol. Using an 21 gauge, 1 in needle, inject the cassette with nuclear extract according to kit instructions.

10. The cassettes were loaded with the C and H nuclear extracts, labeled, and placed together in a 100 mL beaker filled with 50 mL Buffer C and left to dialyze overnight in the fridge.

11. This protocol was followed by the Bradford protocol to determine protein concentration.

**Bradford protein quantification**

1. Gently mix Bradford reagent in bottle and bring to RT.

2. Prepare BSA standards of appropriate concentration in the same buffer as the unknown samples (water can also be used, which we did, according to Sigma protocol). Create two blanks (water), 5 serial dilutions for the standard curve and unknowns for C and H selenium treated nuclear extract, all in duplicate, in 1.5 mL Eppendorf tubes:
5 µL was chosen arbitrarily, as the protein concentration was unknown, but will generally fit within the curve.

3. After combining the reagents in 1.5 mL tubes, vortex gently, incubate 10 min. at RT and transfer to 1 mL polystyrene (for visible wavelengths) cuvettes. Read at 595 nm using the "fixed λ" function on the Beckman DU 640 spectrophotometer.

4. Place the water cuvette in the first slot in the six rack, close the cover, and click 'blank'. Then place the next five cuvettes in, clicking "ReadSamples" twice. Leave both water controls in each time, changing the other 4, always reading twice. Print the results, and label by hand.

5. Using MiniTab, Excel or some other program capable of producing a fitted line plot and equation, enter in the actual [BSA] for the serial dilution and the results from the spectrophotometer. Print the resulting graph and equation. Actual [BSA] in serial dilution:

   (1) 5 µL: 1 µg/µL x 5 µL = 5 µg/1033 µL = 0.00484 µg/µL
   (2) 10 µL: 0.00968 µg/µL
   (3) 15 µL: 0.01452 µg/µL
   (4) 20 µL: 0.01936 µg/µL
   (5) 25 µL: 0.02420 µg/µL

6. Using the equation obtained from the graph: Y = AX + B, where Y is the absorbance, X is the unknown concentration and A and B are given. Compute the average of the four spectrophotometer readings (two samples, two reads) for C and H. This is Y. Solve for X in both cases. Multiply this number by 1033 µL and divide by 5 µL, the volume used. The result is the protein concentration in the nuclear extract in µg/µL.

7. Aliquot both the C and H protein in 10-15 µL aliquots and store at -80 C. Repeated freeze/thaw cycles can degrade the protein.
Estrogen Response Element (ERE) probe

Sequence from Xenopus vitellogenin A2 gene. Estrogen receptor 13-mer palindrome binding site bolded in forward sequence:

46-mer Forward: 5'-TAA CTG TCC AAA GTC AGG TCA CAG TGA CCT GAT CAA AGT TAA TGT A-3'

46-mer Reverse: 5'-TAC ATT AAC TTT GAT CAG GTC ACT GTG ACC TGA CTT TGG ACA GTT A-3'

Preparing radiolabeled probe

1. Warm the H₂O bath to 37°C (cell culture room).
2. Radiolabel the double-stranded probe:
   (1) Combine the following in a 1.5 mL tube:
      2 µL 1 µM double-stranded probe (2 pmoles) (–20°C freezer door)
      a) Mix 20 µL of each single strand (50 µM) with 160 µL dH₂O in
         a 1.5 mL tube (for 5 µM final concentration).
      b) Heat to 75°C (in water bath) for 15 min. and allow to cool slowly (in a beaker with water from the water bath about 30 min. Store at -20 C)
      c) For 2 pmol: dilute 2 µL of 5 µM dsERE with 8 µL dH₂O for 10 µL of 1 µM stock solution. Use 2 µL to obtain 2 pmol.
      2 µL 10 X PNK buffer (–20°C freezer)
      3 µL γ³²P ATP (30 µCi, 10 pmoles)
      1 µL T4 kinase (10 units) (–20°C freezer)
      12 µL H₂O
   (2) Incubate 30 min @ 37°C.
   (3) Dilute reaction mixture to 50 µL with TEN buffer.
   (4) Follow protocol on page 21 of QIAquick nucleotide removal kit instructions to remove free nucleotides.
   (5) Aliquot 2 µL labeled probe into 2 mL scintillation fluid (under dish sink) in a scintillation vial and count on scintillation counter (S288 ESC) to determine radioactive incorporation.
   (6) Determine quantities of labeled (1µL) and cold competitor (dilute 5 µM 1:2 for ~150x) to use in binding reaction.

Plate assembly

1. Assemble clean 16 and 20 cm glass plates, coating one side of one of them with Sigmacote. Use 1 mm spacers and assemble according to BioRad Protean
II instructions, ensuring spacers and glass are flush along the bottom. Tighten down onto one flat gasket only (not two) to minimize leaking.

2. Prepare 50 mL polyacrylamide gel in liquid form and pour along one edge/spacer to avoid bubbles (tilting the assembly to one side facilitates this). If bubbles appear, tap the glass lightly with a hard object (like scissors) until the bubble rises to the top. Fill to about 1 cm from the top.

3. Insert 15 well, 1 mm comb, adding extra liquid gel if necessary to fill to the top. Lay the plates and gel on the side to minimize leaking, if it is occurring.

4. Wait about 20 minutes for gel to completely solidify.

5. Gently remove comb and lock gel in Protean II. Pour 0.5x TBE in the top to ensure there are no leaks and use a pipetman and 10 mL pipet to remove bubbles in the wells with 0.5x TBE.

6. Place central apparatus in buffer-filled well, cap, and pre-run at 150 V with cooling water flowing until mA reach at least 18 (usually starts ~22-24 mA). This usually requires 1-1.5 hrs.

**Binding reaction**

1. Perform binding reaction:
   (1) Prepare buffer B gel shift reaction buffer according to García Pedrero, 2002: See below.
   (2) In 7 microfuge tubes, add gel shift reaction buffer (fridge), poly(dI-dC) (−20°C freezer), nuclear extract (−80°C freezer), cold competitor (-20°C freezer), H$_2$O and antibody according to pipetting scheme below. Incubate 15’ @ 4°C. Add hormone (estrogen at 10$^{-8}$ M) and incubate at 0°C for 10 min if including this step. Then add probe (small freezer) last and incubate 1 hr at 4°C and 30 min at RT.

   **PIPETTING SCHEME:**

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Gel shift buffer (µL)</th>
<th>Poly dI-dC (µg)</th>
<th>150x cold comp (µL)</th>
<th>Probe (pmol)</th>
<th>Nuclear extract (µg)</th>
<th>Antibody (µL)</th>
<th>H$_2$O (µL)</th>
<th>Total (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe1</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0.08*</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
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<td>0</td>
<td>0.08</td>
<td>5</td>
<td>0</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td>H3</td>
<td>10</td>
<td>1</td>
<td>0</td>
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<tr>
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<td>1</td>
<td>0.08</td>
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<td>2</td>
<td>2</td>
<td>21</td>
</tr>
</tbody>
</table>

   * Add probe last.
   #Santa Cruz Biotech recommended 1-2 µL of sc-6820X antibody per 3-10 µg protein with a 15-45 min incubation at RT.
2. Run the gel:
(1) Load each sample into separate wells of the gel. Load 10 µL of 1X bromophenol/xylenol orange blue dye.
(2) Electrophorese @ 100 V (18 mA decreases to 10 or less) ~2 hrs (limits overheating, U shape, and gives sharper bands) until dye front is 2-3 cm from the bottom of the gel, in 0.5x TBE (within the gel and for the running buffer).
(3) Remove and separate plates gently, leaving the gel on one plate. Place moistened Whatman paper on gel, then gently peel from one corner, then from one edge until separated from the second glass plate, taking care not to twist, warp, or tear the gel.
(4) Dry gel @ 80°C under vacuum 60 min. Place plastic wrap on gel surface prior to covering with hard plastic and flexible silicone seal on flat dryer.

3. Expose dried gel to film (dark room) as long as is needed depending on the age of the radioactivity (1-18 hrs). Place dried gel in lead-lined, light-excluding case, taping down if drying caused curling. Place 1-2 sheets of film on the gel in the dark (do not turn on red light if possible) and close the case. Place case in the -80°C freezer and expose the necessary time. Develop film in automatic developer in dark room after allowing it to warm up (~15-20 min., press the 'run' button after turning on the power).

RUN-ON TRANSCRIPTION ASSAY (ref 2)

Preparation of nuclei
1. Pellet up to 1 x 10^7 cells and wash twice with PBS as above for nuclear protein preparation. I used four confluent 25 cm² flasks for both C and H.
2. Follow Panomics Nuclear Extraction Kit protocol with above modifications for nuclear protein isolation up to step seven, completing only the wash with Buffer A Mix. Remove cytosolic protein. Panomics confirmed after this step that nuclei remained.
3. Resuspend pellets in 1 mL glycerol storage buffer for nuclei and store at -80°C.

In-vitro RNA synthesis
1. Gently add 1 volume (100 µL) of 2X transcription buffer to nuclei on ice after adding 2 µL RNaseOUT to top of pellet. Adding 100 µL will make the ATP, CTP, and GTP concentrations each 5x that of the 8 µL biotin-16-UTP. Add 8 µL biotin-16-UTP. Incubate 30 min. at 29°C.
2. To stop reaction, add 6 µL 250 mM CaCl₂, 6 µL RNase-free DNase I and incubate for 10 min at 29°C.
RNA isolation (Invitrogen protocol) (ref 1)

1. Transfer above reaction to new 15 mL conical tubes, using part of Trizol in step 2 below to rinse 1.5 mL tubes used in in-vitro RNA synthesis.
2. Add 2 mL Trizol reagent and lyse nuclei by repetitive pipetting until no longer visible.
3. Incubate the samples for 5 minutes at room temperature.
4. Add 0.2 ml CHCl$_3$/1ml Trizol. Shake tubes vigorously by hand for 15 seconds.
5. Incubate CHCl$_3$ samples for 3 minutes at room temperature.
6. Centrifuge samples for 15 minutes at 12000xg at 2-8°C.
7. Pipet as much of the aqueous phase as possible into fresh 15 mL tubes. Add 0.5 ml 2-propanol/1 ml Trizol to each sample.
8. Centrifuge 2-propanol samples for 10 minutes at room temperature.
9. Centrifuge samples for 10 minutes at 12000xg at 2-8°C.
10. Remove supernatant. Add 1 ml 75% EtOH/mL Trizol to all samples. Vortex samples and centrifuge for 5 minutes at 7500xg at 2-8°C. Vortexing may only separate pellet into smaller chunks, it does not dissolve, as this is a washing step.
11. Pour out 75% ETOH, and allow samples to dry in upside-down tubes for 10-15 minutes. Wipe residual alcohol out of tubes with a kimwipe and spatula.
12. Dissolve RNA pellet in 50 µL DEPC-treated water.
13. Allow to dissolve. Transfer to 1.5 ml tubes and put on ice or in –80°C freezer as quickly as possible.

Binding to magnetic beads

1. Wash 100 µL of Dynabeads M-280 in Dynal Solutions A (two 100 µL washes for 1-3 min.), B (one 100 µL wash) and binding buffer (one 100 µL wash) according to instructions for use for RNA manipulation and nucleic acids immobilization procedures provided with the Dynabeads.
2. Resuspend 100 µL of Dynabeads M-280 in binding buffer at full concentration (10 µg/µL). I also added 1 µL of RNaseOUT to each of the two tubes. Aliquot 50 µL to each of two tubes for C and H RNA.
3. Mix resuspended beads with an equal volume (50 µL) of run-on RNA and incubate 20 min. at 42°C and 2 hours at RT. Beads collected on the bottom of the tubes, though this will not affect results. Enzymes and RNA are much smaller than the beads.
4. Separate beads with the magnetic apparatus supplied by Dynal.
5. Perform two 15 min. washes in 500 µL 15% formamide in 2X SSC, meaning resuspend beads in the wash and allow to incubate at RT, then place in magnet and remove wash. Repeat.
6. Wash 5 min. in 1 mL 2X SSC.
7. Resuspend beads in 30 µL DEPC-treated H$_2$O and store at –20°C. This required some pipetting, as beads were high on the wall of the tube from the last wash.
cDNA synthesis

cDNA synthesis was performed in triplicate, using 10 µL of the above 30 µL final volume for both C and H, for a total of 6 tubes.

1. Combine the following in a 500 µL tube, 1 tube per sample:
   - 1.0 µL random hexamers (50-250 ng)
   - 10 µL run-on RNA (10 µL max)
   - 1 µL 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP, and dTTP at neutral pH)
   - ddH₂O to 12 mL
2. Incubate at 65°C for 5 min. Plunge in ice 2-3 min., spin down condensate briefly.
3. Add:
   - 4 µL 5X first strand buffer
   - 2 µL 0.1 M DTT
   - 1 µL RNaseOUT (40 U/µL)
   - 19 µL total reaction volume
4. Mix contents gently (resuspend the beads with gentle stirring).
5. Incubate at 25°C for 2 min for random hexamers.
6. Add 1 µL (200 units) SSII Reverse Transcriptase, mix thoroughly by pipetting gently up and down.
7. Incubate at 25°C for 10 min (added incubation for use of random hexamers, instead of oligo dT₁₂₋₁₈ (500 µg/mL)).
8. Incubate at 42°C for 50 min.
9. Inactivate enzyme by incubating at 70°C for 15 min.
10. Add 1 µL (2 units) Rnase H, mix thoroughly.
11. Incubate at 37°C for 20 min.
12. Recombine the 3 C and 3 H tubes into one again, for a total of 63 µL each.

Qiagen PCR purification kit columns (Qiagen protocol)

4. Combine 5 volumes of PB buffer with 1 volume of the PCR sample and mix.
5. Place QIAquick spin column in a provided 2 mL collection tube.
6. Apply the sample to the column. Centrifuge 1 min. @ 13,000 RPM on standard tabletop microcentrifuge.
7. Discard flow through and place the QIAquick column back in the same tube.
8. To wash, add 750 µL Buffer PE to the column and centrifuge 1 min.
9. Discard flow through and place the column back in the same tube. Centrifuge the column for an additional 1 min at 13,000 RPM.
10. Place QIAquick column in a clean 1.5 mL centrifuge tube.
11. Elute DNA with 50 µL EB buffer (10 mM Tris-HCl, pH 8.5) to the center of the QIAquick membrane and centrifuge @ 13,000 RPM for 1 min.
12. Add another 50 µL H₂O to each tube for a total of 100 µL.

**Primer Design**

1. **Find Gene Sequence of Interest**
   a) This can be done several ways:
      i) Go to the *Entrez-PubMed* website (www.pubmed.gov)
         (1) Click on “Nucleotide” on the black toolbar at the top of the screen
         (a) Type in the name of the gene of interest and a list of several matches
             (hopefully) will come up. Make sure to pick a sequence for the species
             of interest (homo sapien, etc.) and that match genes you are looking
             for. The number that the gene is listed as is called the *accession*
             *number* and it can be used to search for a particular sequence in any of
             PubMed’s applications. Oftentimes several accession numbers will be
             found for the same gene that are all okay to use.
         (b) It is also possible to click on “Search for Genes” which is found in the
             blue column on the left-hand side of the *Entrez-PubMed* website. This
             will allow searches for genes using LocusLink.
         (i) Type in the name of the gene of interest and a list of several
             matches (hopefully) will come up just like above. Use the same
             criteria to pick the gene of interest. Also, LocusLink allows
             searches in a specific organism’s genome.
   (2) After getting to the *Entrez-PubMed* home page, do a search in PubMed
       using the name of the gene of interest (This is a much longer process).
       This will bring up several articles with the gene of interest listed as a
       keyword.
       (a) Refine the search to “’name of gene of interest’ AND ‘name of
           protocol’”.
       (b) Filter through these articles and find articles that have used primers for
           the gene of interest. Articles may be listed that have the primer
           sequence listed in the article. This takes a long time because access is
           often denied to certain journals/full-text articles online.
       (c) Primers from articles can be used a couple of ways:
           (i) If the article states the bp length of the fragment that will be
               amplified using the primers found, then check that their primers
               will amplify a region of the right gene sequence. Check the primers
               using **BLAST** (instructions below).
           (ii) If the article doesn’t state the bp length of the fragment that will be
                amplified using the primers found then just take the primer
                sequence and enter it into **BLAST** to find accession numbers of the
                gene of interest (instructions below).
   (3) Using **BLAST**:
       (a) To verify if a primer sequence will sit down on the correct gene, use
           BLAST. After entering in the primer sequence, BLAST gives a list of
           all of the different genes (labeled with different accession numbers)
           that the primer sequence will sit down on.
(i) Go to the GenBank website.
(ii) Click on “BLAST” on the black toolbar at the top of the screen.
(iii) Click on “Standard nucleotide-nucleotide BLAST [blastn]”
(iv) Type the primer sequence into the box that has “Search” to the left of it.
(v) Click on the blue “BLAST!” button.
(vi) Click on the blue “Format!” button.
(vii) A list of several genes that the primer matches will come up after waiting a bit. Use this list of genes to either verify that the primers will sit down on the right gene or to find accession numbers for the gene of interest. BLAST lists the genes according to an “E-value.” This E-value corresponds to how complimentary the primer sequence is to a particular gene. Try to use sequences with E-values of .05 or lower.

2. **How to Download Gene Sequences into OMIGA for Primer Design:**
   a) Upon obtaining accession numbers for the genes of interest, click on the accession number (if it is on the screen), or enter the accession number into any of PubMed’s applications and the accession number link will come up.
   b) Upon clicking on an accession number, a taxonomy report will come up on that particular gene.
   c) Click on the down arrow of the box that says “default” and change it to “GenBank” (this box is found next to the box that says “Display” and is on the toolbar at the top of the screen).
   d) Now click on “Save.” This will save the gene in the GenBank format so that OMIGA can read the file when importing it into OMIGA.
   e) A “File Download” textbox will come up. Click “OK” to save the file to a disk.
   f) Rename the file and save it on any hard drive. Make sure to save it as an “.f cgi” file type. It is best to name the file as the name of the gene of interest followed by the accession number. It may be necessary to import several sequences for the same gene and being as specific as possible in naming the file when saving it makes it easier later.
   g) Once the gene sequence is saved on the hard drive, then open OMIGA by going to “Start” then “Programs” then “Omiga” then “OMIGA.”
   h) Open a new project in OMIGA or open an existing project.
   i) Once in OMIGA, then click on “File” then “Import.”
   j) Find the gene that was saved on one of the hard drives and double click on it.
   k) An “Import As Format” textbox will automatically come up. OMIGA will highlight the file format that it recognizes (it should automatically highlight “GenBank Nucleic Acid Format” because it was saved as a GenBank file). Click on “Import.”
   l) A “Name Sequence File” textbox will come up. Enter in a name for the sequence here. When naming the sequence here there is no need to be specific. Just name these sequence as its common name. Name all imported sequences for a given gene the same name. After naming the sequence click on “OK.”


m) The sequence is now imported into OMIGA. From here use the downloaded sequences to design primers.

3. **Primer Design in OMIGA:**
   
a) Open the project in OMIGA.

b) If more than one sequence for a given gene has been imported, then it is important to **align the gene sequences** to identify any differences between them. Do this by highlighting all of the gene sequences needed for the comparison (using the Ctrl key) and by clicking on the “Align Sequences” icon at the top of the toolbar (alternatively, go to “Calculate” and then “Align Sequences.”) A “Clustal W Alignment” textbox will come up. Click on “Align.” A “Save Alignment File” textbox will come up. Save your alignment (save it as the name of the gene you are working with). Make sure to save it as an “.ali” file type.

   i) A “Calculation Progress-Clustal Alignment Progress” textbox will come up. As soon as the alignment is visible, click on the “Alignment” button.

   ii) If there are any ambiguities in the sequences, the “Consensus” sequence will show a gray base. Pick the gene sequence that is the least different from the other sequences to design the primers. There is also the option of using the Consensus sequence to design the primers, but it is easier to just pick one of the sequences already downloaded.

c) Now that the sequence that you would like to use has been chosen, design the primers. Highlight the sequence to use by clicking on it. Then click on the “PCR Primer Pairs” icon which is found on the top toolbar (or you can go to “Search” and then “PCR Primer Pairs.”)

   i) A “Search- PCR Primer Pairs” icon will come up. Create new search parameters (click on “New…”). Name the protocol in the upper left-hand corner.

   ii) For Real-Time PCR, BioRad recommends the following parameters in designing primers:

      1. On the “Individual Primers” tab, set the **Length (bp)** to 18, 20, 22. Set the **% GC Content** to 50, 55, 60. Set the **Tm (C)** to 50, 58, 65. Leave the 3’ clamp residues on and at WSS. (S is code for a G or a C; W is code for an A or a T). The clamp will ensure that on the 3’ end of the primers will have an A or T, then a G or C, then a G or C. Leave all of the other parameters as they are.

      2. On the “Primer Pairs & Products” tab, set the **Length (bp)** to 75, 110, 150. Set the **% GC Content** to 50, 55, 60. Leave the **Tm (C)** at 70, 82.5, 95. Leave all of the other parameters as they are.

      3. When finished adjusting the parameters, click on “OK.” Then click on “Search.” When your primer search is complete, view the primers in a table by clicking on “Table.” This will show a table of all of the possible primers based on the criteria you specified.

      4. If the search failed, that means that OMIGA could not design any primers using the parameters specified. If this happens, then decrease the stringency of the parameters. Start with decreasing the **%GC content** on either tab (“Individual Primers” or “Primer Pairs & Products”). If the
search fails again, then change the 3’ clamp to “SS” rather than “WSS.” If the search fails again, then take off the 3’ clamp altogether. If the search still fails, then slightly change any parameters to help design the primers. If it is this difficult, the primers will probably not be optimal during PCR reactions.

5) If the search does fail, view the “Table” of all possible primers anyway. At the bottom of the textbox there are two other tabs called “Rejected Primers” and “Rejected Pairs.” On these two tabs, OMIGA will list all of the primers that were considered and the reasons they were rejected. This can help to decide which parameters to change.

6) The region of the gene on which to design primers can also be specified. At the bottom of the “Search-PCR Primer Pairs” textbox, click on “Include bp region” and specify the desired starting bp and ending bp. Using this feature drastically limits the number of possible primers. If your searches are consistently failing, it may help not to specify a specific region of the gene.

**Human Primers used for Run-on Transcription Assay and total RNA RT-PCR**

18S Forward: 5’-CGG CTT AAT TTG ACT CAA CAC G-3’
18S Reverse: 5’-CTA AGA ACG GCC ATG CAC C-3’
Cdk2 Forward: 5’-CAA GCC AGT ACC CCA TCT TCG-3’
Cdk2 Reverse: 5’-CAA ATA GCC CAA GGC CAA GC-3’
Cathepsin D Forward: 5’-GCA AAC TGC TGG ACA TCG-3’
Cathepsin D Reverse: 5’-ACC ATT CTT CAC GTA GGT GC-3’
IGFBP10 Forward: 5’-GCC GCC TTG TGA AAG AAA CC-3’
IGFBP10 Reverse: 5’-CTT GCC CTT TTT CAG GCT GC-3’
pS2 Forward: 5’-CAG ACA GAG ACG TGT ACA GTG G-3’
pS2 Reverse: 5’-AGC CCT TAT TTG CAC ACT GG-3’

**Robocycler runs to determine optimum annealing temperatures**

Use the following protocol for each set of primers above with control cDNA made from total RNA. Optimal annealing temperatures will be used for both Run-on Transcription Assay and total RNA gene expression determinations.
Assemble the following into an ice bucket:

- Taq polymerase
- 10 X buffer
- dNTP mix (10µM) or PCR nucleotides
- cDNA pool (dilute about 1:6)
- Primers (10µM each)
- MgCl²
- Purified H²O

**Protocol:**

1. Label a 1.5 ml tube MM for Master Mix.
2. Label 12 PCR tubes with letters coordinating to the primers used and number them starting with 1 through 6 for two sets of primers
3. Add each reagent according to the table below starting with the smallest amount to the largest. If using different primers see below.

<table>
<thead>
<tr>
<th></th>
<th>1 Rxn</th>
<th>8 Rxn</th>
<th>15 Rxn</th>
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<tbody>
<tr>
<td>Taq</td>
<td>0.2 µl</td>
<td>1.6 µl</td>
<td>3 µl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>0.4 µl</td>
<td>3.2 µl</td>
<td>6 µl</td>
</tr>
<tr>
<td>Primers (10µM each)</td>
<td>1.0 µl</td>
<td>8.0 µl</td>
<td>*see below</td>
</tr>
<tr>
<td>10 X buffer</td>
<td>2.0 µl</td>
<td>16.0 µl</td>
<td>30 µl</td>
</tr>
<tr>
<td>cDNA</td>
<td>0.8 µl</td>
<td>6.4 µl</td>
<td>12 µl</td>
</tr>
<tr>
<td>MgCl²</td>
<td>2.4 µl</td>
<td>19.2 µl</td>
<td>36 µl</td>
</tr>
<tr>
<td>H²O</td>
<td>13.2 µl per tube</td>
<td>105.6 µl</td>
<td>198 µl</td>
</tr>
</tbody>
</table>

Add 20.0 µl per tube to make 160.0 µl = 20 µl/8 Rxn tube = 285 µl = 19 µl/15 Rxn tube.

4. Vortex each reagent before adding it to the master mix, **with the exception of the Taq enzyme**. After adding each ingredient, pipette the master mix up and down gently.
5. Once master mix is complete, centrifuge for 5 seconds.
6. Dispense 19µl into of master mix into each PCR tube.
7. Dispense 1µl of primers (10 µl each) into each tube according to how many primers are being sampled. All samples of the same primer should be uniform. If there is no variation of primers add 15µl of primers to the master mix, dispense 20µl into each tube and skip this step.
8. Centrifuge tubes for approx. 5 sec. at a high speed.
9. Add approx. 20 µl of mineral oil to the top of each tube.
10. Centrifuge briefly so mineral oil lies on top of sample layer.
To Run Robocycler:

1. Flip the switch located on the back of the machine. Wait until the computer screen illuminates and the welcome screen comes on.
2. Hit the shift key + 9 (recall program) and recall program #01.
3. Hit the shift key + 1 (set gradient) and choose desired gradient.
4. If necessary, you can use the arrow keys to manually change the values in each window. Be sure to match the annealing gradient in window 1 to the values in window 2, or the annealing gradient block will not be at the correct temperature for the first few cycles.
5. Once all the temperature and cycles are checked to match the table below, hit RUN/STOP and the blocks will heat up. Once the blocks are heated load your samples according to the temperature variation desired and hit ENTER.

**RoboCycler Temperatures**

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<thead>
<tr>
<th>Cycles</th>
<th>Time</th>
<th>Temperature</th>
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</thead>
<tbody>
<tr>
<td>1 (window 1)</td>
<td>4 minutes</td>
<td>95°C</td>
</tr>
<tr>
<td>35 (window 2)</td>
<td>30 seconds</td>
<td>95°C</td>
</tr>
<tr>
<td></td>
<td>30 seconds</td>
<td>Annealing Gradient</td>
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<tr>
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<td>45 seconds</td>
<td>72°C</td>
</tr>
<tr>
<td>1 (window 3)</td>
<td>7 minutes</td>
<td>72°C</td>
</tr>
<tr>
<td>1 (window 4)</td>
<td>indefinite</td>
<td>6°C</td>
</tr>
</tbody>
</table>

**Determining Annealing Temperature**

1. Pour a 2% agarose mini gel with a 7 tooth comb and allow it to harden.
2. Add 4 µL of 6x Loading dye to each sample and mix for a total of 24 µL. Spin briefly.
3. Add 10 µL to each well and 3 µL 100 bp ladder to the last lane.
4. Run in 1x TAE buffer at 100 V for ~60 minutes until dye front is 1 cm from the bottom.
5. Soak gel 10 min in Ethidium Bromide, then 10 min in water. Visualize, label and print on Alpha Imager. Expose at about 1-1.5 sec, adjusting black and white until a good picture of your bands is visible.
6. Use the temperature at which the band is the brightest for the annealing temperature in LightCycler runs.
**LightCycler Run**

1. Assemble the following into an ice bucket:
   - Taq polymerase
   - 10 X buffer
   - dNTP mix (10 µM) or PCR nucleotides
   - cDNA:
     - undiluted pool (10 µL C and 10 µL H)
     - 1:2 pool (10 µL undiluted and 10 µL dH₂O)
     - 1:4 pool (10 µL 1:2 and 10 µL dH₂O)
     - 1:8 pool (10 µL 1:4 and 10 µL dH₂O)
     - 1:16 pool (10 µL 1:8 and 10 µL dH₂O)
     - C Se 1:6 dilute (2 µL cDNA and 10 µL H₂O)
     - H Se 1:6 dilute (2 µL cDNA and 10 µL H₂O)
   - SYBR green 1:2000 dilute (add 1 µL 10000x to 49 µL H₂O for 1:50, then 1 µL of 1:50 dilute to 39 µL H₂O for 1:2000 dilution)
   - Primers (10 µM or 25 µM each)
   - MgCl₂
   - BSA (1 mg/mL)
   - Purified H₂O

2. Label a 1.5 ml tube MM for Master Mix

3. Add each reagent according to the table below starting with the smallest amount to the largest. With the exception of the SYBR green which is to be diluted and added in after the H₂O and the cDNA which will be added individually at the end.

<table>
<thead>
<tr>
<th></th>
<th>1 Rxn</th>
<th>20 Rxn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq</td>
<td>0.16 µl</td>
<td>3.2 µl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>0.40 µl</td>
<td>8 µl</td>
</tr>
<tr>
<td>Primers (10 µM each)</td>
<td>1.00 µl (10 µM) or 0.40 µl (25 µM)</td>
<td>20 µl or 8 µl</td>
</tr>
<tr>
<td>10 X buffer</td>
<td>2.00 µl</td>
<td>40 µl</td>
</tr>
<tr>
<td>cDNA</td>
<td>2.00 µl</td>
<td>*add individually</td>
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<tr>
<td>MgCl₂</td>
<td>3.20 µl</td>
<td>64 µl</td>
</tr>
<tr>
<td>BSA</td>
<td>5.00 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>5.10 µl or 5.70 µl</td>
<td>102 µl or 114 µl</td>
</tr>
<tr>
<td>SYBR green (1:2000 dilute)</td>
<td>1.14 µl</td>
<td>22.8 µl</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>20.00 µl per capillary</th>
<th>360 µl = 18 µl per cap.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>20 Rxn (2 extra)</td>
<td></td>
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</tbody>
</table>

4. Vortex each reagent before adding it to the master mix, **with the exception of the Taq enzyme.** After adding each ingredient, pipet the master mix up and down gently.

5. Once master mix is complete, centrifuge for 5 seconds.
6. Place 18 capillaries in the LightCycler centrifuge adaptors.
7. Dispense 18 µl of the MM into each capillary.
8. Dispense 2 µl of cDNA into each capillary coordinating to the numbers below.

   1) 1:1 cDNA pool   13) C Se 1:6 dilute
   2) 1:1 cDNA pool   14) H Se 1:6 dilute
   3) 1:2 cDNA pool   15) H Se 1:6 dilute
   4) 1:2 cDNA pool   16) H Se 1:6 dilute
   5) 1:4 cDNA pool   17) H₂O
   6) 1:4 cDNA pool   18) H₂O
   7) 1:8 cDNA pool
   8) 1:8 cDNA pool
   9) 1:16 cDNA pool
  10) 1:16 cDNA pool
  11) C Se 1:6 dilute
  12) C Se 1:6 dilute

9. Centrifuge capillaries in their adaptors for 30 sec at 2500 rev/min.
10. Cap each capillary with white cap.
11. Gently twist capillaries into LightCycler capillary holders, starting with number 1 on the carousel. THEY EASILY BREAK!!

To run LightCycler

1. Double click on NEW LIGHT CYCLER SOFTWARE 3.5.3
2. Click RUN on main menu
3. Allow machine to self test, once finished click OK.
4. Go to file and click open. Select program and click OPEN.
5. Under Experiment, click on Amplification. Adjust the middle Target Temperature to desired annealing temperature.
6. Change setting/names of carousel slots as needed under Edit Samples, depending on whether it is a standard, unknown, or negative control.
7. Click RUN and save experiment.
8. After amplification, once the flow reaches about 100 or the graph starts to slope off click END PROGRAM and allow machine to go to next program, melting curve analysis. DO NOT click on EXIT RUN.

Quantification

Once experiment has finished, Data Analysis will automatically open.

1. At the top left click on Select a Program and choose Melting Curve Analysis.
2. Choose Melting Curve on the top of the screen. Check to make sure all DNA denatured at the same temperature and that there is only one peak on the
bottom graph. Deselect those that have additional peaks (primer dimers), and print the results (Ctrl-P).

3. Close that screen and click on Select a Program again. Choose Amplification.
4. On the top of the screen click Quantification.
5. The program defaults to Second Derivative Maximum (SDM) method. Select sample #1 only. Using two straight edged instruments, such as rulers, line up vertically at the crossing point for the selected sample with one ruler and horizontally with the other ruler from the point of amplification to determine the fluorescence at which the second derivative was at its maximum for that sample.
6. Repeat for all other samples individually until you have, hopefully, a consensus. Remember or write down any samples that do not have a similar fluorescence as the others. They should be in the range of 0.5-1.0 fluorescence different from the others, either up or down.
7. Select Fit points and check Step 3: Analysis. Ctrl-deselect any samples that had more than one peak in the Melting Curve analysis.
8. Select Step 2: Noise Band and adjust the Noise Band line a few tenths of fluorescence below the optimal value you determined in the SDM analysis. It should be in the amplification phase of the run.
9. Select Step 3 again and set the fluorescence band right at the value determined by the SDM method. Also check the 'Show Fit Points' box, but leave the number of points at 2.
10. With the 'Show Fit Points' box selected, cross marks will show which measurement points the program is using to determine the slope. The fluorescence band should be between these two fit points for as many samples as possible. If not, return to Step 2 and adjust the noise band until the fluorescence band, at maximum SDM efficiency, lies between all or nearly all fit points. Be sure to re-adjust the fluorescence band when you return to Step 3, as it will move when you move the noise band.
11. Carefully look at your samples and replicates, remembering which SDM points were not like the others. If a Calculated Concentration is very different from its replicates, or a point on the standard curve is way off, hopefully the SDM point was also different, giving you more leverage to eliminate that point. Your goal is to get an r-value of at least -0.95 or closer to -1.0, and as many matching replicates as possible in your unknowns.
12. Use Ctrl + P to print the window, making sure the screen shows all of the Grouped Means, Calculated Concentrations and Crossing Points.

TOTAL RNA RT-PCR

RNA isolation from LNCaP cells

1. RNA was isolated as above, according to the Invitrogen protocol, except that 2.5 mL TRIzol was used per 25 cm² flask, and the RNA pellet was re-dissolved in 100 µL RNAse free H₂O per 1 mL TRIzol.
RNA quantification

Quantification of RNA using spectrophotometry allows you to determine how much RNA you have isolated as well as how free of protein contaminants it is.

1. Make sure that spectrophotometer is on. The UV lamp must be turned on and must have 10 min. to warm up. It must have a chance to cool for at least 45 min before being turned on again.

2. Use Beckman capillaries for UV readings. One capillary is for each sample. A 'blank' cuvette will contain only water. Use the capillary holder in the single rack. Optimal position was found to be at 56.1 mm.

3. Pipet 7 µL of each sample onto a weigh boat and use capillary action to draw the sample into the capillary (the capillary holds about 5 µL). Seal with a small amount of Critoseal and wipe clean a few times with a Kimwipe. Repeat for each sample.

4. Take cuvettes to spectrophotometer. Select 'nucleic acids.'
   o Open lid and place BLANK cuvette (containing water only) in capillary holder.
   o Close the lid.
   o Press the 'Blank' button. Wait for display to stabilize at 0.000. Click 'ReadSamples' a few times.
   o Open the lid. Remove blank and place sample in holder.
   o Close the lid. Click 'Readsamples' a few times until the numbers stabilize or rise and fall so an average is obtained.
   o Repeat for other samples.

5. Calculate the 260/280 ratio. A 'very clean' sample will have a 280/260 ratio of between approximately 1.8 and 2.0. Alternatively, a 280/260 should be between 0.4 and 0.55.

6. Calculate the concentration of RNA in each sample using the following equation. (Note that the equation only uses the OD_{260}.)

   \[ \text{[µg/µl]} = \left( \text{OD}_{260} - \text{OD}_{320} \right) \times 0.8 \]

   \text{bold}\ = \ conversion\ coefficient\ for\ RNA

\text{cDNA Synthesis}

Carried out as above for Run-on Transcription Assay, except the cDNA was diluted from 48 µL after the Qiagen clean-up to 192 µL (added 48 µL H₂O, then 96 µL H₂O) to ensure enough supply for all LightCycler runs.
RoboCycler and LightCycler runs

Both were carried out as above. For the RoboCycler, optimum annealing temperatures were determined for all genes using total RNA-produced cDNA, whether the primers were used here or for the Run-on Transcription Assay. LightCycler protocol is the same, except that cDNA from total RNA was used.

Statistical Analysis

For EMSAs, t tests were used to compare the relative mean band densities for control and high Se-treated samples.

Statistical analysis was performed using a randomization test with the concentrations calculated by the LightCycler software. These calculated concentrations were compared to those derived using the method of Pfaffl (3) and found to be nearly identical. To examine relative gene expression, the mean of the replicates of the gene of interest was normalized by dividing by the mean of the replicates for 18S rRNA. For each gene the normalized value for the high Se group was divided by the normalized value for the adequate Se group to give a ratio for relative gene expression.

To determine statistical significance, all replicates of calculated concentrations for the gene of interest in control Se-treated cells were randomized together with all replicates for 18S rRNA in the same cells. The same randomization was done with all replicates for the gene of interest and for 18S in high Se-treated cells. Following this randomization the same calculations as described above were performed to derive a final ratio expressing relative gene expression. This randomization and recalculation was repeated 1000 times, and the number of recalculated ratios greater than and less than the original calculated ratio were tallied to determine the probability of obtaining the original ratio simply by chance. The null hypothesis - that the treatment had no effect on gene expression - would predict an original ratio of 1.0. The only assumption made about the data was that of independence, as each sample was run in a separate capillary. In this randomization model no assumptions are made about the distribution of the original calculated concentrations or the values calculated from the randomized data. A more detailed description of these statistical methods is in preparation.

REFERENCES--MATERIALS AND METHODS


SOLUTIONS AND BUFFERS

Pedrero Buffer B

1 M HEPES-KOH (pH 7.9, adjusted with KOH, 15 mL, 3.575 g HEPES in H₂O made separate)
2 M KOH (15 mL, 1.683 g KOH in H₂O made separate)

0.020 M HEPES-KOH, pH 7.9 (300 µL above)
0.01 M MgCl₂ (0.0143 g)
0.001 M EDTA (30 µL 0.5 M)
0.1 M KCl (0.112 g)
0.0002 M phenylmethylsulfonyl flouride (0.000523 g)
0.0002 M DTT (30 µL 0.1 M)
0.5% Nonidet P-40 (75 µL)
10 % glycerol (1.5 mL)
protease inhibitors (~15 µL)
Bring up to 15 mL with dH₂O

PBS

10x stock solution:
1370 mM NaCl (80 g)
27 mM KCl (2 g)
43 mM Na₂HPO₄·7H₂O (11.5 g)
14 mM KH₂PO₄ (2 g)
Bring up to 1 L with d H₂O, ensuring pH is ~7.4, then filter sterilize

PBS (500 mL):

137 mM NaCl (4 g)
2.7 mM KCl (0.1 g)
4.3 mM Na₂HPO₄·7H₂O (1.45 g)
1.4 mM KH₂PO₄ (0.12 g)
Bring to 400 mL with dH₂O, adjust pH to ~7.4, bring to 500 mL & filter sterilize

5x TBE electrophoresis buffer

0.445 M Tris base (54 g)
0.445 M Boric acid (27.5 g)
2 mL 0.5 M EDTA, pH 8.0
Bring upto 1 L with dH₂O

5x TAE electrophoresis buffer

1 M Tris·acetate (24.2 g Tris base and 5.71 mL glacial acetic acid)
50 mM EDTA (3.72 g Na₂EDTA·2H₂O)
Bring up to 1.0 L with dH₂O, adjusting, if necessary, to pH ~8.5

**TEN buffer**

10 mM Tris-HCl (100 µL 1.0 M Tris-HCl, pH 8.0)
100 mM NaCl (1 mL 1 M NaCl)
1 mM EDTA (20 µL 0.5 M EDTA)
Bring up to 10 mL with dH₂O

**Bromophenol Blue**

0.25% bromophenol blue (0.025 g)
20% Ficoll (2.0 g)
Bring up to 10 mL in dH₂O

**Bromophenol Blue/Xylenol Orange**

0.25% xylenol orange
Add 0.0025 g xylenol orange to 1 mL bromophenol blue solution

**Glycerol storage buffer for nuclei**

0.05 M Tris-HCl, pH 8.3 (750 µL)
40% glycerol (6 mL)
0.005 M MgCl₂ (0.00714 g)
0.0001 M EDTA (3 µL 0.5 M EDTA)
Bring up to 15 mL in dH₂O

**40% acrylamide (39 acrylamide:1 bisacrylamide)**

19.5 g acrylamide
0.5 g bisacrylamide
Bring up to 50 mL with dH₂O
degas under vacuum several minutes
lasts 30 days if stored in the dark and refrigerated

**50 mL EMSA gel: 5% acrylamide**

5 mL 5x TBE
6.25 mL 40% acrylamide (39 acryl:1 bisacryl)
38.75 mL dH₂O
last minute:
400 µL 10% APS (ammonium persulfate, made fresh)
40 µL TEMED
2% agarose gel

2 g agarose added to 100 mL 1x TAE buffer (make large quantity each time)
Heat and swirl to mix/pour

2X Transcription buffer (run-on transcription assay RNA synthesis):

0.2 M KCl (0.0149 g)
0.02 M Tris-HCl, pH 8.0 (20 µL 1 M Tris, pH 8.0)
0.005 M MgCl₂ (100 µL 0.05 M MgCl₂)
0.004 M DTT (40 µL 0.1 M DTT)
4 mM each ATP, GTP, and CTP (40 µL ea of 100 mM solutions)
0.2 M sucrose (0.0685 g)
20% glycerol (200 µL)
Bring up to 1 mL with dH₂O

Dynal Solution A

0.1 M NaOH
0.05 M NaCl

Dynal Solution B

0.1 M NaCl

Dynal Binding buffer:

0.01 M Tris-HCl, pH 7.5 (10 µL 1 M Tris, pH 7.5)
0.001 M EDTA (2 µL 0.5 M EDTA)
2 M NaCl (0.1169 g)
Bring up to 1 mL with RNase-free H₂O

SSC wash:

15% formamide (2.25 mL formamide in 12.75 mL 2x SSC)
2X standard saline citrate (SSC) (1.5 mL 20x SSC and 13.5 mL RNase-free H₂O)

20X SSC (15 mL):

3 M NaCl (2.630 g)
0.3 M Na₃citrate·2H₂O (1.323 g)
Bring up to 15 mL with RNase-free H₂O and adjust pH to 7.0

CaCl₂

0.25 M CaCl₂ (0.0368 g CaCl₂·2H₂O)
Bring up to 1 mL with RNase-free H₂O

**Panomics Buffer A Mix**

1 mL 1x Buffer A  
10 μL 100 mM DTT  
10 μL Protease Inhibitor Cocktail  
40 μL 10% IGEPAL

**Panomics Buffer B Mix**

147 μL 1x Buffer B  
1.5 μL Protease Inhibitor Cocktail  
1.5 μL 100 mM DTT
PURCHASED REAGENTS

**Amersham Biosciences, Piscataway, NJ**

Poly (dIdC)*Poly(dIdC) (10 A260 units, ~500 µL)--product # 27788001

**ATCC, Manassas, VA**

RPMI-1640 medium--item no. 30-2001
LNCaP FGC Prostate Cancer; Human--item no. CRL-1740

**Beckman Coulter, Inc., Fullerton, CA**

Spectrophotometer capillaries--part no. 514262

**Bio-Synthesis Incorporated, Lewisville, TX**

Estrogen response element and other primers--custom ordered

**Cambrex Bio Science Walkersville, Inc., Walkersville, MD**

0.025% Trypsin/0.265 mM EDTA solution (100 mL)--prod. code CC-5012

**Corning Costar Corp., Cambridge, MA**

25 cm2 Flask, tissue culture treated, polystyrene with phenolic style cap--item no. 430372

**Dynal Biotech LLC, Brown Deer, WI**

Dynabeads M-280 Streptavidin (10 mg/mL, 2 mL)--item no. 112.05

**Fisher BioReagents, Fairlawn, NJ**

Ribonuclease H (50 U, 2U/µL, 25 µL)--cat. no. BP3215-1
T4 Polynucleotide Kinase (100 U, 10U/µL, 10 µL)--cat. no. BP3212-1

**HyClone, Logan, UT**

Defined Fetal Bovine Serum (Lot # APA20504)--purchased from, catalog # SH30070

**Invitrogen Life Technologies Corp., Carlsbad, CA**

Random Primers (9 A260 U, 3 µg/µL)--cat. no. 48190-011
Ribonuclease H (30 U, 2 U/µL, 15 µL)--cat. no. 18201-014
RNaseOUT Recombinant Ribonuclease Inhibitor (5000 U, 40 U/µL, 125 µL)--cat. no. 10777-019
Superscript II RNase H Reverse Transcriptase (10000 U, 200 U/µL, 50 µL)--cat. no. 18064-014
TRIzol Reagent (100 mL)--cat. no. 15596-026

Panomics, Inc., Redwood City, CA

Nuclear Extraction Kit (10 reactions)--cat. no. AY2002

PerkinElmer Life Sciences, Inc. Boston, MA

Adenosine 5'-triphosphate (gamma-32P, 100 UCi, 3700 KBQ, 10 µL)--part no. NEG002A100UC

Pierce, Rockford, IL

Slide-A-Lyzer Dialysis Cassette (10,000 MWCO, 0.1-0.5 ml capacity, 10 ea)--product # 0066415

Promega, Madison, WI

Magnesphere Magnetic separation stand (2-hole, 1.5 mL vial)--item no. Z5332

QIAGEN Sciences, Inc., Germantown, MD

QIAquick Nucleotide Removal Kit (50)--cat. no. 28304
QIAquick PCR Purification Kit (50)--cat. no. 28104

Roche Diagnostics Corp., Indianapolis, IN

Biotin-16-uridine-5'-triphosphate (250 nmol, 10 mM, 25 µL)--cat. no. 1388 908
DNase I, RNase-free (10000 U, 10 U/µL, 1000 µL)--cat. no. 776 785
Ribonucleotide set (4x20 µMol, 200 µL, 100 mM lithium salt)--cat. no. 1 277 057

Santa Cruz Biotechnology, Santa Cruz, CA

Estrogen receptor beta antibody (N-19X)--item no. sc-6820 X

Sigma-Aldrich Corp., St. Louis, MO

(±)-α-tocopherol (5 g)--cat. no. T-3251
Bradford reagent (500 mL)--cat. no. B6916
Penicillin-Streptomycin Solution (100×) Stabilized (10,000 U/ml penicillin and 10 mg/ml streptomycin, 100 mL)--cat. no. P4333
RPMI-1640 Medium (with L-glutamine and without phenol red and sodium bicarbonate)-
-cat. no. R-8755
Sigmacote (100 mL)--cat. no. SL-2
APPENDIX C

RAW DATA

Run-on transcription assay
* unusable data

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<thead>
<tr>
<th>Gene</th>
<th>control calculated concentration</th>
<th>high calculated concentration</th>
<th>noise band</th>
<th>fluorescence crossing point</th>
<th>group</th>
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<th>Percent</th>
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<tbody>
<tr>
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Frequency Missing = 433

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</tr>
</thead>
<tbody>
<tr>
<td>0.95315059</td>
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Data set used was Cathepsin D-total RNA group 1

<table>
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<tr>
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<th>Percent</th>
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<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
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</tr>
</thead>
<tbody>
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Frequency Missing = 791

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</tr>
</thead>
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<tr>
<td>1.1396873797</td>
<td>1001</td>
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Data set used was Cathepsin D-total RNA group 2

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<th>Percent</th>
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<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>little</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
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<th>Cumulative Percent</th>
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<tr>
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Frequency Missing = 487

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<th>Cumulative Percent</th>
</tr>
</thead>
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<td>0.9743910102</td>
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Data set used was Cdk2-ROTA group 1

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</thead>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>100.00</td>
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<th>Cumulative Percent</th>
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</tr>
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Frequency Missing = 505

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</tr>
</thead>
<tbody>
<tr>
<td>original</td>
<td>0.997754091</td>
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<td>1001</td>
<td>100.00</td>
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Data set used was Cdk2-ROTA group 2

<table>
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<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
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<td>222</td>
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Frequency Missing = 779

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</tr>
</thead>
<tbody>
<tr>
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<td>778</td>
<td>100.00</td>
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</table>

Frequency Missing = 223

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<tr>
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<td>1001</td>
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Data set used was Cdk2-total RNA group 1

<table>
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<th>Percent</th>
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<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
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<td>881</td>
<td>100.00</td>
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</tbody>
</table>

Frequency Missing = 120

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<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>119</td>
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Frequency Missing = 882

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</tr>
</thead>
<tbody>
<tr>
<td>1.1082037777</td>
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<td>1001</td>
<td>100.00</td>
</tr>
</tbody>
</table>
Data set used was hpS2-total RNA group 1

<table>
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<tr>
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<th>Percent</th>
<th>Cumulative Frequency</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
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Frequency Missing = 252

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<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
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<td>100.00</td>
<td>251</td>
<td>100.00</td>
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</tbody>
</table>

Frequency Missing = 750

<table>
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<th>Percent</th>
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<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0875649059</td>
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</tbody>
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Data set used was hpS2-total RNA group 2

<table>
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<tr>
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<th>Frequency</th>
<th>Percent</th>
<th>Cumulative Frequency</th>
<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
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</tbody>
</table>

Frequency Missing = 33

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<tr>
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<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
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<td>100.00</td>
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Frequency Missing = 971

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<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2403714445</td>
<td>1001</td>
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</tbody>
</table>
## COMPILED STATISTICAL DATA

### Run-on transcription

<table>
<thead>
<tr>
<th>gene</th>
<th>calculated mean ratio</th>
<th>p-value for &lt;1</th>
<th>group (replicates)</th>
<th>p-value for &gt;1</th>
<th>weighted average</th>
<th>weighted probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP10</td>
<td>0.78813 0.006</td>
<td>1 (9)</td>
<td></td>
<td>0.994</td>
<td>0.9947</td>
<td>0.385</td>
</tr>
<tr>
<td>IGFBP10</td>
<td>1.30399 0.957</td>
<td>2 (6)</td>
<td></td>
<td>0.043</td>
<td>0.9947</td>
<td>0.385</td>
</tr>
<tr>
<td>Cdk2</td>
<td>0.99775 0.504</td>
<td>2 (7)</td>
<td></td>
<td>0.496</td>
<td>0.9947</td>
<td>0.385</td>
</tr>
<tr>
<td>Cdk2</td>
<td>0.78915 0.222</td>
<td>2 (9)</td>
<td></td>
<td>0.778</td>
<td>0.88041</td>
<td>0.347</td>
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<tr>
<td>Cathepsin D</td>
<td>0.81179 0.288</td>
<td>1 (5)</td>
<td></td>
<td>0.712</td>
<td>0.89427</td>
<td>0.372</td>
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<td>Cathepsin D</td>
<td>0.95315 0.432</td>
<td>2 (7)</td>
<td></td>
<td>0.568</td>
<td>0.89427</td>
<td>0.372</td>
</tr>
</tbody>
</table>

### Total RNA

<table>
<thead>
<tr>
<th>gene</th>
<th>calculated mean ratio</th>
<th>p-value showing sig. for &lt;1</th>
<th>group (replicates)</th>
<th>p-value showing sig. for &gt;1</th>
<th>weighted average</th>
<th>weighted probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP10</td>
<td>1.23069 0.966</td>
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<td></td>
<td>0.034</td>
<td>1.23069</td>
<td>0.034</td>
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<tr>
<td>Cdk2</td>
<td>1.10820 0.881</td>
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<td>0.119</td>
<td>1.10820</td>
<td>0.119</td>
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<tr>
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<td>0.97439 0.486</td>
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<td>0.514</td>
<td>1.02949</td>
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<tr>
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<td>1.02949</td>
<td>0.413</td>
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<td>1 (3)</td>
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<td>0.030</td>
<td>1.12577</td>
<td>0.196</td>
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<td>1.12577</td>
<td>0.196</td>
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</tbody>
</table>
FIG 4. **LightCycler Results Example.** Representative of typical output obtained from the LightCycler. Calculated concentrations used above in the raw data section come from the second to last column on the left portion of the image.