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Breast Cancer is a tumor of the mammary gland epithelium affecting about 176,000 women in the western hemisphere and killing about 50,000 people every year in the United States. A substantial body of epidemiological, experimental, and clinical evidence indicates that exposure to the natural hormones, estrogen, progesterone and prolactin, which are important for the normal development and function of the breast tissue, play a major role in the growth of breast cancer cells and may even contribute to the development of breast cancer. The specific aims of our proposal are to develop highly sensitive PCR methods for rapid, specific, diagnosis of breast cancers on the basis of their estrogen- and progesterone receptor expression. The results we have obtained indicate the feasibility of identifying and quantitating various forms of the hormone receptors in a small amount of tumor tissue by molecular assays.

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Robert E. Taylor, M.D., Ph.D. 28 Feb 97

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TABLE OF CONTENTS

COVER i

REPORT DOCUMENTATION PAGE ii

FOREWORD iii

TABLE OF CONTENTS iv

INTRODUCTION 1

SPECIFIC AIMS 2

BODY 2

CONCLUSIONS 6

REFERENCES 7

FIGURES 8

A. INTRODUCTION

Breast cancer is a tumor of the mammary gland epithelium affecting about 176,000 women in the western hemisphere and killing about 50,000 people every year in the United States. A substantial body of epidemiological, experimental, and clinical evidence indicates that exposure to the natural hormones, estrogen, progesterone and prolactin, which are important for the normal development and function of the breast tissue, play a major role in the growth of breast cancer cells and may even contribute to the development of breast cancer.

1. Prognostic factors in breast cancer. Once diagnosed with breast cancer, therapeutic approaches, survival rate and disease recurrence depend upon the expression of the prognostic factors, hormone receptors, growth factor receptor superfamily and proteins involved in invasive metastatic phenotype in the tumor tissues.

2. Hormone receptors in the prognosis and therapy of breast cancers. The most important among prognostic factors are the hormone receptor, estrogen-, and progesterone receptors (ER and PgR). Estrogen receptor. The presence of ER in tumors indicates a good prognosis and the patients respond to anti-estrogen therapies. The expression of various molecular forms of ER was studied in several breast cancer cell lines and tissues. The results have revealed very interesting findings. Analysis of the estrogen receptor mRNA has shown that it undergoes alternate / inaccurate splicing in the tumor tissues giving rise to several aberrant (variant) forms of the receptor molecules[1]. These studies raise the possibilities of various species of ER which have exon deletions/truncations in the 1) estrogen binding region (exons 4-8), 2) DNA binding region (exons 2-3) and 3) other portions of the receptor molecule. In T47D cells, several variant mRNA species and their protein products lacking exons 2, 3, or 7 representing 25% of the ER mRNA were recognized[2]. **To study the effect of the above exon deleted\truncated (variant) species, cDNAs corresponding to wild type and variant forms were expressed in cell lines and the estrogen-, and DNA binding properties were evaluated. It was found that the variant species interfere with the normal wild type ER for its binding with estrogen response elements (ERE) on the DNA- and hormone binding and estrogen induced gene transcription presumably by heterodimer formation[3].**

3. Current methods of estrogen receptor evaluation and their disadvantages. The presence of ER in the tumor tissues are currently evaluated by immunochemical methodologies in clinical laboratories. While these provide information on the presence of ER, they are 1) very cumbersome, 2) time consuming, 3) not highly sensitive, 4) require a large sample 5) **do not yield quantitative information**, 6) not suitable to evaluate in fine needle aspirations and

not yield quantitative information, 6) not suitable to evaluate in fine needle aspirations and 7) very expensive to the patient. **Most importantly, immunohistochemical assays cannot distinguish between the wild type and variant forms of ER, therefore, cannot predict hormone therapy responders precisely. Because of the therapeutic considerations, there is an urgent need to rapidly quantitate and get a profile of wt and vt of ER to predict hormone therapy responders and disease prognosis.**

The essence of our proposal is to develop highly sensitive cost effective quantitative PCR assays to measure a multitude of hormone receptors and other diagnostic and prognostic factors in breast cancers.

4. Development of new prognostic assays for estrogen receptor. In our grant, we proposed to develop highly sensitive, rapid, cost-effective PCR based methods to quantitate ER the prognostic factors in a small amount of clinical samples. The developed assays could be used virtually in every clinical lab to diagnose and evaluate prognosis of breast cancers. The significance of this assay is that patients could then be identified who are most likely to respond to hormone therapy and that the overall prognosis may be assessed.

B. SPECIFIC AIMS.

The specific aims of our proposal are to develop highly sensitive PCR methods for rapid, specific, diagnosis of breast cancers on the basis of their estrogen- and progesterone receptor expression. To achieve these goals we proposed to conduct the following.

1. Quantitation of wild type and variant forms of estrogen receptor in estrogen responsive cell lines: a) RNA extraction, b) reverse transcription, c) primer selection, d) PCR, e) relative quantitation and f) absolute quantitation.
2. Correlation of the transcript number with functionally active estrogen receptor molecules in estrogen responsive cell lines: a) estrogen binding activity and b) DNA (ERE) binding activity
3. Quantitation of wild type and variant types of estrogen receptor in breast tumor tissues
4. Comparison of estrogen receptor mRNA levels and functionally active protein levels in patient samples
5. Application of RTP asssay to predict prognosis and hormone therapy response in patients.

C. BODY OF THE REPORT (STUDIES AND RESULTS).

In the first granting period, we reported the following: 1) procuring the ER positive and negative cell lines and conditions for growing them 2) standardized RNA isolation procedures 3) conducted reverse transcriptions on the isolated RNA 4) Designing the primers pairs for ER which can amplify DNA binding, hormone binding and hinge regions of ER 5) testing of the primers on cloned plasmid ER cDNA to optimizing the PCR conditions for amplifying the

above mentioned regions of ER and 6) designing a competitor for ER cDNA which would allow us to quantitate the various exon portions of ER.

During the second year of granting period, we have focussed on achieving our goal of quantitating the wild type and variant forms of estrogen receptor in breast cancer cell lines.

1. Quantitation of estrogen receptor transcripts. Having established the PCR amplification of estrogen receptor, we began the experiments on the quantitation of wild type and variant types of estrogen receptor transcripts by competition assay. Briefly, the quantitation of the transcripts is achieved by precisely determining the number of specific RNA transcripts by template competition[4]. In this approach, two templates which are specific for the same primers are used in the PCR reactions. The templates are amplified at the same efficiency and the ratio of the two PCR products will reflect the starting ratio of the two template DNAs. This means that if the amount of a competitor DNA is known then the amount of unknown template can be precisely determined by measuring the two PCR products. This requires that the products generated by the two different templates must be distinguishable from each other.

a. Designing and cloning of a competitor plasmid for the absolute quantitation of estrogen receptor. We have designed a competitor plasmid as described in the Fig.1. As described in the Fig.1 each set of primers used to amplify the DNA- (exons 4-8) and hormone binding regions (exons 1-4) flanks an unique HindIII site within exon 4. A 250 bp HindIII fragment from bacteriophage lambda DNA was inserted into the site. This construct is used as a competitor to quantitate estrogen receptor transcripts. When this is used as a competitor template in a PCR reaction for the estrogen receptor, it generates a product that is 250 bp larger than the normal product and easily distinguishable on an agarose gel.

b. Testing of the competitor plasmid. Using the above competitor with primer pairs which can amplify exons 1-4, 4-6, and 4-8, we generated PCR products which are 250 bases higher than the normal product. The results obtained using the full length ER plasmid cDNA (pIC-ER) and the competitor plasmid (pSG-ER-lambda) with the primer pairs of exons 1-4, 4-6, 4-8 and 1-8 are shown in the Figure 2. Lanes 1 standards, lane 2, competitor plasmid only, and lanes 3-14 represent exon 4-6 the products of competition assay with pIC-ER-F and pSG-ER-lambda. These results clearly demonstrate that the cloned competitor plasmid generates a 250 bp larger fragment therefore could be used in competition assay.

c. Identification of ER variants which have exon deletions in various breast cancer cell lines. After establishing various procedures as described above, we went to amplify ER exons in breast cancer cell lines. We describe here amplification of the DNA binding region (exons 4-8) in the breast cancer cell lines, MCF-7 and T47D. When we amplified the above exons, we observed atleast three species of DNA, 866 bp, the expected wild type species, 800 bp fragment and a 750 bp fragment in MCF-7 cell line (Figure 3A, lane 2) and somewhat similar pattern in T47D cell line (Figure 3B, lane 2). To identify these low molecular weight forms, they were purified from the primers and subjected to digestions with restriction enzymes, Bgol2, NcoI and PstI. Exon five has an unique NcoI site and exon 6 has

two unique sites recognized by BgolII and PstI. If there are no deletions in these two exons, it is expected that they will be cleaved by the above enzymes. Our results indicate that NcoI cleaved the PCR amplified DNA from both cell lines into 524 bp, 300 bp which are expected from the wild type and additional species of size 400 bp. The enzyme PstI digestion gave 450 and exon. BglII digestion gave 478, 388 bp bands which are expected and a faint 300 bp band (Figure 3A, lane 3. and Figure 3B, lane 3). All these observations are consistent with the previously reported results that exon 7 is deleted in the above cell lines. We are currently in the process of sequencing the variant to confirm the exon 7 deletion.

d. Establishing the quantitation assay using cloned ER cDNA competitor. To confirm the ability of competition assay to accurately measure ER transcripts, a competition assay was performed using known amounts of both the competitor template and the cloned normal ER cDNA gene. ER primer pair which amplifies exons 4-6 was used and the number of copies of ER cDNA was kept constant at 10^7 while the competitor concentration ranged from 10^9 (Fig.4, lane 1) copies to 10^4 (lane 10) copies using half-log dilutions. As seen in Figure 4, the amount of competitor needed to generate equal ratios of the two PCR products is between 10^7 copies (lane 4) and $10^{7.5}$ copies (lane 5). This clearly demonstrates the feasibility of quantitating ER using this approach.

d. Designing and construction of a competitor template DNA for the quantitaion of glyceraldehyde 3-Phosphate dehydrogenase (GAPDH) transcripts. to normalize the number of ER and PgR transcripts in the tumor tissues, we propose to quantitate Glyceraldehyde 3-Phosphate gene and express the number of copies of ER with reference GAPDH. The expression of GAPDH does not change in transformed tissues. We will quantitate the number of GAPDH copies by the competition assay as described above. For the competition assay, we have designed and cloned a competitor as for ER. To clone GAPDH competitor, first a 500bp portion of the GAPDH cDNA[5] (bases # 181-680 counting from the translational start site) was amplified by RTPCR from RNA extracted from human breast cancer cell line MCF-7 using primers which attached an upstream EcoR1 restriction enzyme site and a downstream BamHI sites of pBluescript II SK+(Strategene). Next a 210 bp lambda DNA fragment (from bases 1920 to 2130 of the lambda sequence) was amplified by PCR using the primers which attached NcoI site within the 500 bp piece of GAPDH cDNA above. We have already cloned a GAPDH competitor using the procedure described above. In a given tumor tissue, the GAPDH copy numbers will be quantitated and the number of ER transcripts will be expressed with respect to the number of GAPDH tramscipts.

2. Progesterone receptor quantitation in breast cancer. In the second granting period, we have also begun quantitation of PgR. We have designed primers to amplify various forms of PgR and established the optimum conditions for amplifying them.

a. Design and testing of PCR primers which can specifically amplify various forms of progesterone receptor mRNA. The genomic and cDNA sequences of the human progesterone receptor have been published. Because of the observations that progesterone receptor is expressed as atleast three, A, B and a short C forms in breast cancer tissues[6], we

have designed several primer sets so that all the three forms can be amplified and quantitated. The primer sets are given in the Table.1. We have tested the above primers first with cloned human progesterone receptor cDNA gene, pSG-PgR2, which contains the coding sequences for A form and an additional sequences of 814 from B form. The plasmid was obtained from Dr. P. Chambon's laboratory at EMBL. The primer set PgRB1 and PgRC2 will amplify B form only, the set PgRA1 and PgRC2 is used for the amplification of both A and B forms and the primer pair PgRC1 and PgRC2 for the amplifications of all three A, B and C forms. Our results showing the amplification of all the above forms using plasmid cDNA is shown in Fig.5. Lanes 1-6 are the amplification products of C form with primers C1 and C2 with Mg^{++} ion concentration 1-6 mM. Lanes 7-10 are the products of A form with primers A1 and C2 Mg^{++}ion concentration in the range of 1-4, and lanes 11-13 are amplification products of B form with B1 and C2 Mg^{++}ion concentration in the range of 1-4.

TABLE 1: PCR PRIMERS FOR THE HUMAN PROGESTERONE RECEPTOR (hPgR)

PRIMERS	LOCATION
PgRB1 Sense 5'ACCAGCTCTTGCTGCCTGTTT 3'	1153-1174
PgRA1 Sense 5'TACCTTGTGGCCGGTGCCAAC 3'	1953-1974
PgRC1 Sense 5'GGGCAGCACAACTACTTATGT3'	2531-2553
PgRC2 anti-sense 5'TGAAAGAAGTTGCCTCTCGCC 3'	2907-2928

b. Amplification of PgR transcripts in breast cancer cell lines. After establishing the optimum PCR conditions to amplify various forms of PgR, we have tested the primers on cDNA prepared by reverse transcribing the RNA isolated from MCF-7 and T47D cell lines. Figure 6 describes the amplification of PgR using the primers C1 and C2 in MCF-7 and T47D and GAPDH. Lanes 1 has standards, lanes 2 and 5 contain the PCR products of GAPDH, lanes 3 and 4 are in MCF-7 and lane 6 in T47D. For the quantitation of PgR transcripts in breast tumors, we will use the same strategy as described for ER. This requires that a competitor plasmid needs to be designed and cloned as for ER and GAPDH. This is described below.

d. Designing and cloning of a competitor for quantitation of PgR. A 296 bp lambda DNA fragment (from bases 1920 to 2216 of the lambda was amplified by PCR using primers which attached BclI sites to both ends. This fragment was subcloned into the unique BclI restriction site within the plasmid hPgR2[7] which contains the cDNA for the human progesterone receptor form A. We have cloned the competitor plasmid and tested it using primer pair C1 and C2. The results are described in Fig.7. Lane 1 shows the 100 bp standards, lane 2 contain the PCR product with the competitor plasmid (pSG-PgR-lambda) and lane 3 and

4 contain the PCR products with plasmid, pSG-PgR.

D. CONCLUSIONS

The results we have obtained indicate the feasibility of identifying and quantitaing various forms of the hormone receptors in a small amount of tumor tissue by molecular assays.

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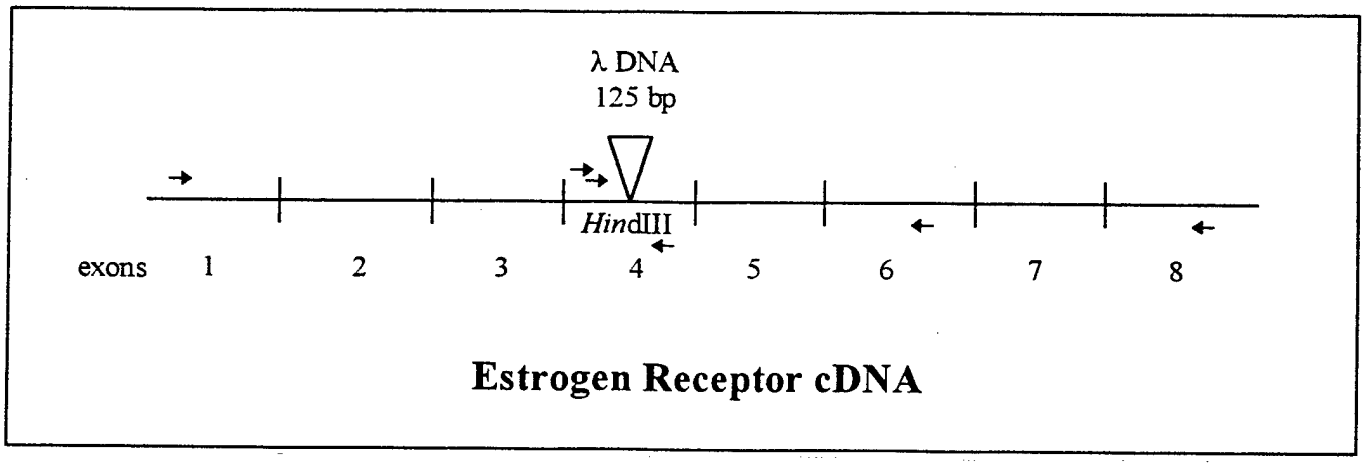

Fig.1 Schematic representation of primers and lambda DNA insertion into ER cDNA gene.

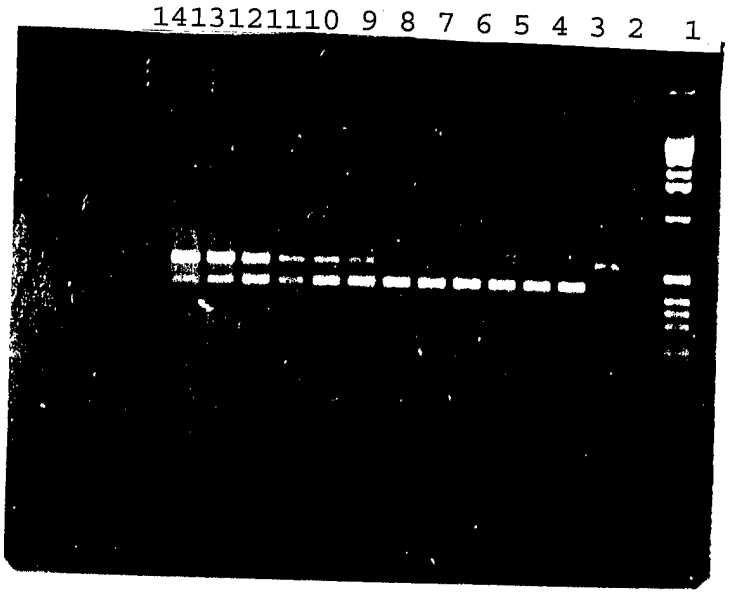

Fig.2 Competitive PCR of ER plasmid pIC-ER-F and pSG-ER-F-lambda with primers which can amplify ER exons 4-6.

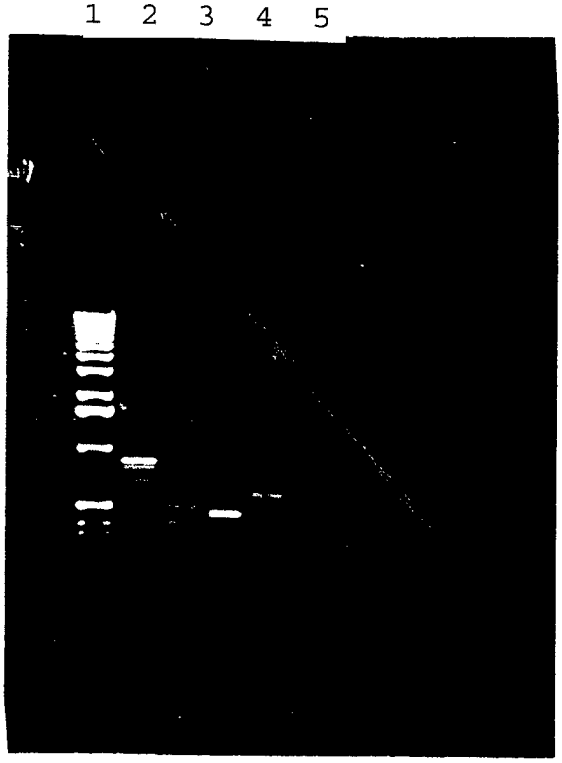

Fig. 3A. PCR amplification of ER exons 4-8 in MCF-7 cells and restriction digestion.

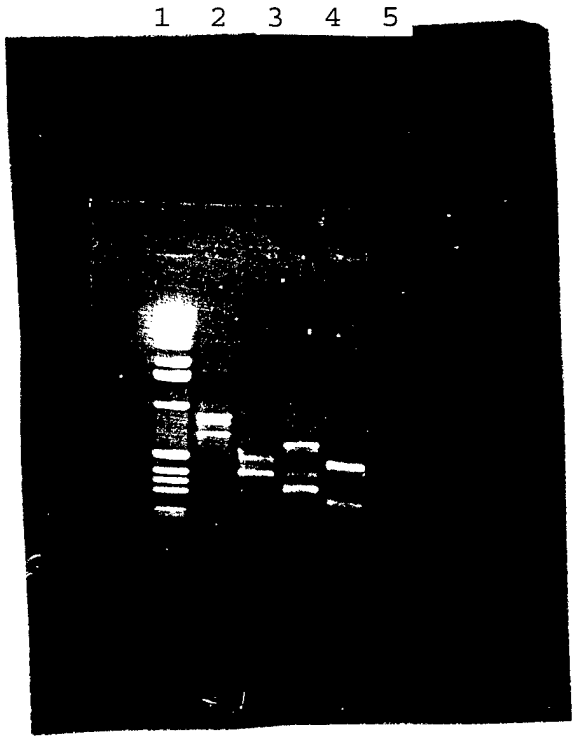

Fig. 3A. PCR amplification of ER exons 4-8 in T47D cells and restriction digestion.

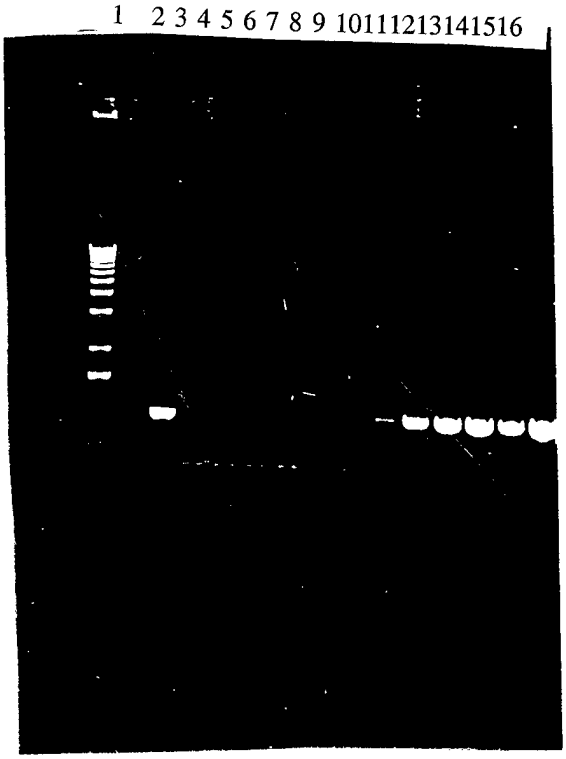

Fig.4. Quantitation of ER exons 4-6 in MCF-7 cells by template competition

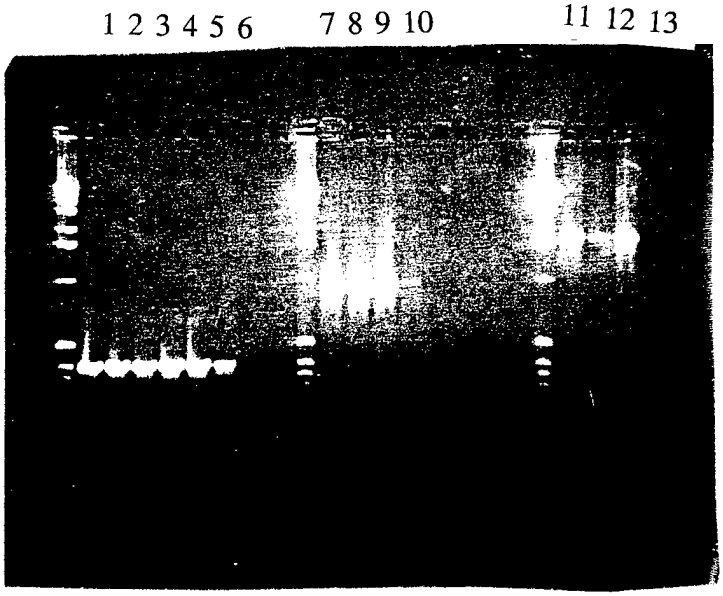

Fig. 5. Amplification of Progesterone receptor A, B, and C forms by PCR using plasmid cDNA

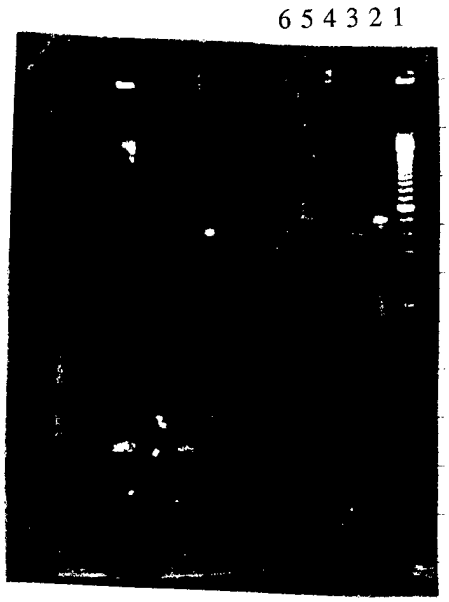

Fig.6. Amplification of Progesterone receptor C form in MCF-7 and T47D cells.

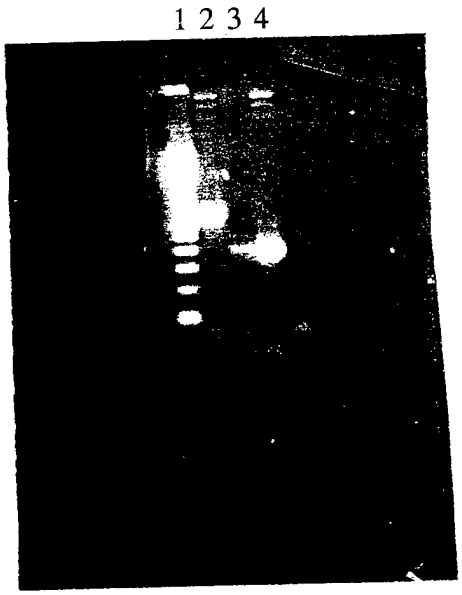

Fig.7 Competitive PCR of Progesterone receptor plasmid pSG-PgR and pSG-PgR-lambda with primers which can amplify C form.