Endocrine, *vol. 22, no. 2, 101–111, November 2003* 0969–711X/03/22:101–111/$25.00

Molecular Assays to Profile 10 Estrogen Receptor Beta Isoform mRNA Copy Numbers in Ovary, Breast, Uterus, and Bone Tissues

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Estrogens regulate various biological processes in a diverse range of reproductive and nonreproductive tissues through two genetically distinct but structurally related high affinity nuclear receptors, the estrogen receptor alpha and beta (ERα and ERβ). The physiological significance of the presence of two ERs that have redundant functions is not known. Several unique properties of ERβ together with its distinct expression patterns are considered to be, in part, the basis for diverse functional actions of estrogens and opposing actions of selective estrogen receptor modulators (SERMs) in different tissues. To understand how relative expression levels of two ERs correlate to seemingly dissimilar actions of estrogens and SERMs, quantitative methods are required that can precisely measure the levels of every isoform. Previously, methods to quantify eight ERα isoforms have been described [Poola I. (2003) *Anal. Biochem.* 314, 217–226]. In this article, real-time PCR-based molecular assays are described that can distinguish and quantify as low as 100 copies of 10 ERβ isoform mRNAs, the ERβ1, ERβ2, ERβ4, ERβ5, and ERβ exon 2Δ, exon 3Δ, exon 4Δ, exon 5Δ, exon 6Δ, and exons 5–6Δ. Each isoform mRNA is quantified using a specific primer pair and a 5'FAM (carboxy-fluorescein)- and 3'TAMARA (6-carboxy tetraethyl-rhodamine)-labeled probe and in comparison with a standard curve constructed with known copy numbers of its respective reverse transcribed cRNA. The devised assays were applied to profile 10 ERβ isoforms in four estrogen-sensitive tissues—ovary, breast, uterus, and bone. The sensitivity of detection of each isoform in these tissues varied from picograms to nanograms of reverse-transcribed total RNA depending on the isoform and the tissue. The results presented also show that each tissue has a distinct profile of 10 isoform mRNAs. Interestingly, ERα-negative breast cancer cell lines and tumors expressed significant amounts of ERβ isoforms suggesting that mitogenic stimulation by estrogen exists in these tissues. Bone tissues expressed several isoforms, although wild type was not present. In addition to the assay development, evidence is presented to demonstrate for the first time that ERβ4 and ERβ5 are full length receptors, contrary to previous reports that they are short receptors of exon 7–8. It is expected that the methods described here will significantly contribute to delineating the functional roles of various ERβ isoforms and in conjunction with ERα isoform profiling, will highly facilitate designing of individualized tissue specific therapies to treat estrogen-related pathologies.

Key Words: ERβ wild type; ERβ isoforms; ERβ splice variants; quantitative real-time-PCR; splice targeted primers; splice variant mRNA quantification; breast; ovary; uterus; bone and breast cancer.

Introduction

The estrogens mediate a wide variety of complex biological processes in a diverse range of tissues from reproductive tracts of both males and females, mammary tissues, to nonreproductive tissues that include cardiovascular, skeletal, immune, nervous, gastrointestinal, and urinary tracts *(1)*. In addition to maintenance of normal functions of the above systems, mitogenic actions of estrogens are implicated as a cause of developing malignant breast tumors *(2)*. Hence, there is an immense interest to understand the molecular mechanisms of actions of this hormone in a broad range of seemingly unlinked biological processes.

Recent progress in the understanding of molecular mechanisms of estrogen signal transduction pathways has revealed that different cells respond to the same hormone in a different manner because of the differences in the expression levels of several proteins involved in mediating estrogen action *(3)*. The most important players in the estrogen-mediated cellular signaling are the estrogen receptors (ERs). Two functionally related but genetically distinct ERs, the ERα and ERβ, have been identified that mediate estrogen action. The estrogen-bound ERs are transcription factors, which upon binding to target gene promoters activate the transcription of genes under estrogen control *(4)*. The two ER isoforms share homologous sequences in the DNA- and hormone-binding regions (96% and 60% respectively)

Received June 10, 2003; Revised July 8, 2003; Accepted July 8, 2003.

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(5), and therefore interact with identical DNA response elements and exhibit similar binding affinity profiles with a number of natural and synthetic ligands *(6)*. The physiological significance of the presence of two ERs that seem to have redundant functions is not known. However, several unique structural and functional properties of ERβ, together with distinct tissue expression patterns, suggest that it plays a regulatory role in estrogen signaling. It lacks an N-terminal activation function (AF-1) domain *(5)* and the C-terminal activation function domain (AF-2) that interacts with co-activators and co-repressors functions differently from the similar ERα domain *(7)*. In addition, the ERβ does not activate transcription of genes regulated at AP-1 promoter site when bound with ERα agonists because of lack of AF- l domain. Instead, many ERα blockers that function as antagonists of ERβ transcriptional activity on ERE-containing promoters, act as potent agonists at AP-1 responsive elements *(8)*. A number of in vivo and in vitro studies have also suggested that ERβ (1) can function independently of ERα in estrogen action *(9)*, (2) has constitutive transcriptional activity by activating EREs of target gene promoters in the absence of ligand, (3) has a modulatory role on ERα-mediated transcriptional activity *(10–12)*, and (4) when expressed together with ERα, it forms heterodimers *(13)*. These studies indicate that the ERβ plays a regulatory role, in addition to being an independent receptor, in estrogen-mediated cellular signaling.

The expression pattern of ERα and ERβ also suggests that different biological response to estrogen in different tissues could be directly related to differential levels of these receptors. Although most of the estrogen responsive tissues express both receptors, one form seems to predominate over the other depending on the tissue type. For example, ERα was shown to predominate in mammary tissue and female reproductive tissues, whereas ERβ appears to predominate in ovary, male reproductive tissues, and nonreproductive organs such as spleen, lung, hypothalamus, thymus, and cardiovascular and skeletal system *(14)*. Because a majority of target tissues express both receptors, binding to ligands, recruitment and interaction with co-repressors/co-activators, promoters, and an overall responsiveness to a particular ligand are most likely to be determined by ERα:ERβ ratio. Therefore, the ability to precisely determine the levels of each receptor will contribute considerably to our understanding of how an expression profile of the two receptors correlates with biological response. The outcomes will have significant impact on designing individualized therapies for estrogen-related pathologies.

The expression levels of receptor protein molecules are traditionally detected and quantified by either immunohistochemical or Western blotting methods. Although these conventional methods are well suited for a research set up, they cannot be applied to limited amounts of clinical samples such as breast cancer tissues that express both receptors *(15, 16)*. In these cases, detection and quantification of receptor mRNAs may be preferred, because it requires considerably smaller amounts of tissue sample. Furthermore, the currently used immunohistochemical method in clinical laboratories cannot distinguish between several isoforms of the receptors. In the current report, quantitative real-time PCR-based assays are described to distinguish and quantify 10 ERβ isoforms, the ERβ1, ERβ2, ERβ4, ERβ5, ERβ exon 2Δ, ERβ exon 3Δ, ERβ exon 4Δ, ERβ exon 5Δ, ERβ exon 6Δ, and ERβ exons 5–6Δ. The devised assays were applied to profile the above isoforms in four estrogen-sensitive tissues, ovary, breast, uterus and bone tissues and breast cancer cell lines and tissues. The results show that each tissue has a unique profile of ERβ isoforms. It is expected that these methods together with ERα isoform quantifying methods will significantly aid in profiling all the known estrogen receptor isoforms and our understanding of how a qualitative and quantitative ER isoform composition in a tissue correlates with the response /resistance to a particular estrogenic/anti-estrogenic ligand.

Results and Discussion

In recent years it has become clear that ERα-blockers or selective estrogen receptor modulators (SERMs) act differently in different target organs. The SERMs Tamoxifen and Raloxifen oppose estrogen action in breast but mimic its biological effects in bone tissues. The opposing actions of these molecules in different tissues is thought to depend on relative levels of ER isoforms and the availability of several cellular factors that include the types and levels of co-activators and co-repressors *(1,3,4)*. Recent reports that Tamoxifen resistance in ERα-positive breast tumors correlated with elevated levels of ERβ *(17,18)* but not with other co-factors suggested that ERβ is an important factor in determining tissue-specific actions of SERMs. Although the molecular interactions that confer this resistance are not clear, it is likely, in part, due to agonistic effects of ERα-blockers when bound to ERβ on AP-1 promoter sites. Increased ERβ to ERα levels may result in increased agonistic actions of SERMs. In all likelihood the relative expression levels of ERβ and ERα could dictate the tissue-specific agonistic/antagonistic actions of SERMs. Therefore, to design new individualized effective endocrine treatments for estrogen-related abnormalities such as breast cancer and osteoporosis, it is important to precisely profile all ER isoform levels in a given tissue.

Previously, molecular approaches to determine the expression levels of eight ERα isoform mRNA levels were described *(19)*. The current report describes the design, testing, and application of real-time PCR-based approach for profiling ERβ isoform in a number of estrogen-sensitive tissues. In addition to the wild type, the ERβ1, methods are also described to profile nine isoforms that have been iden-

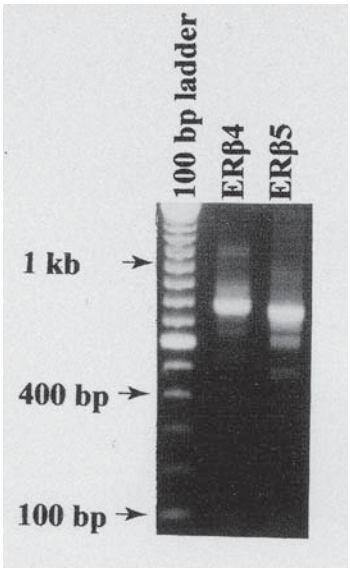

Fig. 1. Amplification of ERβ4 and ERβ5 coding sequences from ovary cDNA. The coding sequences for ERβ4 and ERβ5 were amplified using a sense primer in exon 4 (ERβ 2S) and isoform-specific antisense primers (ERβ4.A and ERβ5.A, respectively) (Table 1) in exon 8 as described in *Methods* section. The sense primer together with ERβ4-specific antisense primer amplified a 775 bp product and the ERβ5-specific antisense primer amplified a 745 bp product. These two products were cloned, sequenced (see Fig. 2), and identified as coding sequences of ERβ4 and ERβ5, providing the first evidence that they are full-length receptors.

tified thus far in various tissues. They are ERβ2, ERβ4, ERβ5, ERβ exon 2Δ, ERβ exon 3Δ, ERβ exon 4Δ, ERβ exon 5Δ, ERβ exon 6Δ, and ERβ exons 5–6Δ. The ERβ2 and ERβ3 are full-length molecules, share identical sequences with ERβ1 from exon 1 to 7, but diverge in exon 8 *(20)*. In contrast, ERβ4 and ERβ5 were described as partial sequences spanning from a portion of exon 7 and distinct exon 8 sequence *(20)*. The functional properties were studied for ERβ2, and it was shown to form heterodimers with ERβ1 and ERα and mediate estrogen signaling although several fold weaker than ERβ1 *(21)*. Among the exon deletion splice variants, functional activities have been demonstrated for exon 3Δ and exon 5Δ *(22,23)*, although the presence of other exon deletion mRNAs have been reported in several tissues *(24)*.

In the current study, for the purpose of constructing plasmids for cRNA synthesis, ERβ isoform sequences were amplified using a sense primer in exon 4 and isoform-specific antisense primers in exon 8 from human ovary cDNA. During that process, unexpectedly 775 bp and 745 bp products were amplified with isoform-specific antisense primers for ERβ4 and ERβ5, respectively (Fig. 1). Upon cloning the two ERβ4 and ERβ5 products into pCR®2.1-TOPO vector and sequence analysis using three different primers, coding sequences were identified that are expected of full-length receptors (Fig. 2). Both products gave similar sequences from exon 4 to exon 7 and diverged in exon 8 similar to ERβ2 and ERβ3. These data provide the first evidence that both ERβ4 and ERβ5 could be full-length receptors and are expressed in four estrogen-responsive tissues, and breast cancer cell lines and tissues along with other eight isoforms.

To quantify every isoform by real-time PCR, primer pairs were first designed and tested for their specificity. For exon deletion splice variants, recently described "splice targeted primers" *(24,25)*, were applied to amplify their mRNAs as separate gene populations (Table 1). Every primer pair was tested for specificity using two different templates: (1) its respective reverse-transcribed cRNA and (2) cDNA prepared from ovary total RNA by conventional PCR. The results obtained with 10 primer pairs are presented in Fig. 3. The ERβ1, ERβ2, ERβ4, ERβ5, ERβ exon 2Δ, ERβ exon 3Δ, ERβ exon 4Δ, ERβ exon 5Δ, ERβ exon 6Δ, and ERβ exon 5–6Δ primer pairs generated expected PCR products of size 186 bp, 176 bp, 206 bp, 177 bp, 200 bp, 283 bp, 273 bp, 241 bp, 229 bp, and 241 bp, respectively, without non-specific amplification or cross amplification.

After establishing the specificity of all the primers, the sensitivity of the primers together with 5'FAM- and 3'TAMARA-labeled probes were tested using ERβ isoform DNAs prepared by reverse transcription of various copy numbers of their respective cRNAs as described in the *Methods* section. Sensitivity was tested with transcripts ranging from 10^2 to 10^9 copies of reverse-transcribed cRNA. The results obtained for 10 isoforms, presented in Table 2, indicate that each primer pair detected either 10^3 copies or 10^2 copies at 50% ramp rate. Representative amplification plots and standard graphs generated for ERβ1, ERβ2, ERβ4, and ERβ5 shown in Fig. 4 indicate that the four primer pair and probe sets generated amplification plots with as few as 10^2 copies. The C_T values varied only ± 0.5 cycle for any given copy number and isoform when repeated several times over a period of 6 mo. The standard graphs show that the assays are linear between 10^2 and 10^9 copies indicating that the quantifications could be performed in this range. These results also show the efficacy of the designed primer pairs and probes in detecting very low copy numbers of ERβ isoform mRNAs by reverse-transcription real-time PCR.

Next, the primer pair and probe sets for ERβ1, ERβ2, ERβ4, and ERβ5 and exon deletion splice variants were tested for their sensitivity to detect cDNAs prepared from breast, ovary, and uterus tissues. For this, the tissue cDNAs, reverse-transcribed from 50 ng of total RNA, were serially double diluted and amplified by real-time PCR as described in the *Methods* section. The sensitivity is defined as the dilution (minimum amount of reverse-transcribed total RNA) that gives the detectable amplification plot. The results obtained for 10 isoforms, presented in Table 2, indicate that the sensitivity of detection varied from isoform to isoform

Fig. 2. Nucleotide sequence of 775 bp and 775 bp products amplified with a sense primer in exon 4 and ERβ4- and ERβ5-specific antisense primers, respectively. The above products were cloned into pCR®2.1-TOPO vector and each identified by sequence analysis using three different primers (ERβ1S, ERβ 2S, and ERβ4.A for ERβ4 and ERβ1S, ERβ 2S, and ERβ5.A for ERβ5) (Table 1). **A**: Sequence obtained for ERβ4. **B**: Sequence obtained for ERβ5. The exon demarcations are indicated. Two of the primers used for sequencing each product (**A**: ERβ1S and ERβ4.A; **B**: BRβ 1S and ERβ5.A) are bold.

and in different tissues. Representative amplification plots generated for ERβ1, ERβ2, ERβ4, and ERβ5 with serially double diluted cDNAs from breast, ovary, and uterus are shown in Fig. 5. In breast tissue, the ERβ1, ERβ2, ERβ4, and ERβ5 sequences could be detected with 1:16 (3.125 ng), 1:16 (3.125 ng), 1:4 (12.5 ng), and 1:64 (0.78 ng) diluted samples, respectively. In ovary, the dilutions were 1:256 (0.19 ng), 1:128 (0.39 ng), 1:32 (1.56 ng), and 1:256 (0.19 ng), respectively. In the uterus the dilutions were 1:2 (25 ng), 1:8 (6.25 ng), 0, and 1:16 (3.125 ng), respectively (Table 3). The minimum amount of reverse-transcribed total RNAs required for detecting other isoforms in the above tissues are also shown in Table 3.

The devised assays were applied to profile 10 ERβ isoforms in four estrogen-sensitive tissues—the ovary, breast, uterus, and bone—and one ERα-positive (MCF-7) cell line, one ERα-negative breast cancer cell line (MDA-MB-231), one ERα-positive breast cancer tissue, and one ERα-negative breast cancer tissue. For the quantifications, the amount of cDNA used was generally about 5–10 times higher than the minimum amount required to detect that particular isoform depending on the tissue (Table 3). The number of copies

Table 1
Primers and Probes for Quantitation of ERβ Isoforms by Real-Time PCR

Wild Type (ERβ1)
Sense (ERβ1S) 5'tttgggtgattgccaagagc3' (Position, exon 7, bp 1411–1430)
Antisense (ERβ1.A) 5'agcacgtgggcattcagc3' (Position, exon 8, bp 1580–1597)
Probe 1: FAM 5'cctcccagcagcaatccatgcg3'TAMARA (Position, exon 7, 1438–1460)
ERβ2
Sense (ERβ1S) 5'tttgggtgattgccaagagc3'
Antisense (ERβ2.A) 5'gtcactgctccatcgttgct3' (Position, exon 8, bp 1907–1887)
Probe 1: FAM 5'cctcccagcagcaatccatgcg3'TAMARA
ERβ4
Sense (ERβ1S) 5'tttgggtgattgccaagagc3'
Antisense (ERβ4.A) 5'gtctgggttttatatcgtctgcaa3' (Position, exon 8, bp 271–294)
Probe 1: FAM 5'cctcccagcagcaatccatgcg3'TAMARA
ERβ5
Sense (ERβ1S) 5'tttgggtgattgccaagagc3'
Antisense (ERβ5.A) 5'cacttttcccaaatcacttcacc3' (Position, exon 8, bp 243–265)
Probe 1: FAM 5'cctcccagcagcaatccatgcg3'TAMARA
ERβ exon 2Δ
Sense (ERβ SX 1/3) 5'acacaccttacctgtaaacagl/3 gaca3' (Positions, exon 1, bp 440–460, and exon 3, bp 634–637)
Antisense (ERβ 2A) 5'gctgctcgtcggcacttc3' (Position, exon 4, bp, 813–795)
Probe 2: FAM 5'tgggtaccgccttgtgcggag3'TAMARA (Position, exon 4, bp 770–790)
ERβ exon 3Δ
Sense (ERβ SX2/4) 5'tttaaaagaagcattcaag2/4 gctc3' (Positions, exon 2, bp 615–633, and exon 4, bp 751–754)
Antsense (ERβ 3A) 5'aactccttgtcggccaactt3' (Position, exon 4, 996–1015)
Probe 3: FAM 5'tagtgctcaccctcctggaggctga3'TAMARA (Position, exon 4, bp 901–925)
ERβ exon 4Δ
Sense (ERβ SX3/5): 5'gaatggtgaagtgtg 3/5 gctttg3' (Positions, exon 3, bp 736–750 and exon 5, bp 1051–1056)
Antisense (ERβ 4A) 5'atcatggccttgacacagaga3' (Position, exon 6, bp 1289–1300)
Probe 4: FAM 5'aggtgttaatgatggggctgatgtggc3'TAMARA (Position, exon 5, bp 1108-1134)
ERβ exon 5Δ
Sense (ERβ 2S) 5'cggcaaggccaagagaag3' (Position, exon 4, bp 824–841)
Antisense (ERβ AX6/4) 5'gcatttcccctcatcc 6/4 cggga3' (Positions, exon 6, bp 1190–1205 and exon 4, bp 1046–1050)
Probe 3: FAM 5'tagtgctcaccctcctggaggctga3'TAMARA
ERβ exon 6Δ
Sense (ERβ 3S) 5' catgatgatgtccctgaccaa3' (Position, exon 4, bp 977– 997)
Antisense (ERβ AX7/5): 5'gaccagagggtacatac 7/5 ctgt3' (Positions, exon 7, bp 1340-1324 and exon 5, bp 1189–1186)
Probe 4. FAM 5'aggtgttaatgatggggctgatgtggc3'TAMARA
ERβ exon 5–6Δ
Sense (ERβ 2S) 5'cggcaaggccaagagaag3' (Position, exon 4, bp 824–841)
Antisense (ERβ AX7/4) 5'ccagagggtacatac 7/4 cgggaa3' (Positions, exon 7, bp 1340–1324 and exon 4, bp 1045–1050)
Probe 3: FAM 5'tagtgctcaccctcctggaggctga3'TAMARA

of every isoform in a given amount of sample was determined in comparison with a standard graph constructed with known copy numbers (10^2–10^9 copies) of its respective reverse-transcribed cRNA as described in *Methods* section. A standard graph was constructed every time quantitation of a test sample was performed. The number of copies of every isoform was normalized to 10^{10} copies of GAPDH.

The results obtained for each isoform in each tissue/cell line tested and presented in Table 4 indicate that ERβ5 was the most abundant isoform in each tissue tested. This suggests ERβ5 may play an important role in estrogen-mediated signaling in target tissues. Breast tissues expressed 9 of the 10 isoforms. Ovary expressed the highest amounts of all the 10 isoforms among the tissues analyzed. Bone tissue expressed ERβ2 and ERβ5, although ERβ1 was not observed, indicating that these isoforms may be the major mediators of estrogen action in this tissue. The ERβ exon 3Δ that was shown to have ligand binding and transcriptional properties *(21)* was detected only in ovary as reported previously by conventional PCR *(24)*. Interestingly, ERα-negative breast tumor and the cell line, MDA-MB-231, expressed all the isoforms at the same level as the ERα-positive tumor

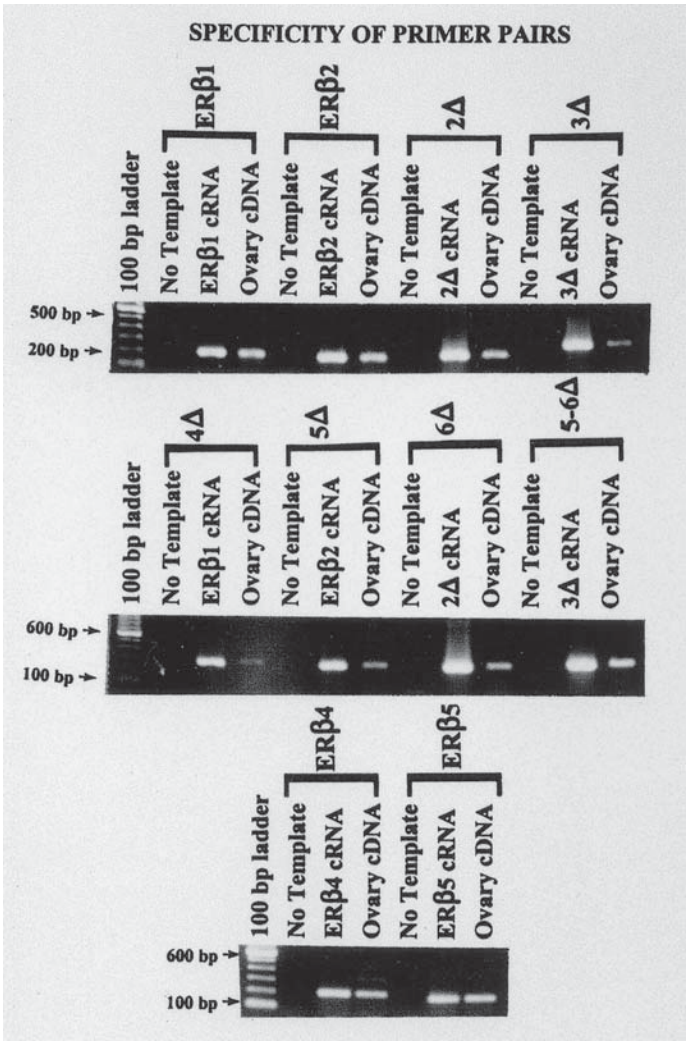

Fig. 3. Establishing the specificity of the designed primer pairs by conventional RT PCR. The primer pairs described in Table 1 were tested for their specific amplification using two different templates: (1) reverse-transcribed complementary RNA (cRNA) that was generated by in vitro transcription of cloned ERβ isoform nucleotide sequences and (2) cDNA prepared from ovary. The PCRs conditions were as described in the *Methods* section. The primers designed for ERβl, ERβ2, ERβ4, ERβ5, ERβ exon 2Δ, exon 3Δ, exons 4Δ, exon 5Δ, exon 6Δ, and exons 5–6Δ (Table 1) specifically amplified the expected products of sizes, 186 bp, 176 bp, 206 bp, 177 bp, 200 bp, 283 bp, 273 bp, 241 bp, 229 bp, and 241 bp, respectively, without cross amplification or nonspecific amplification.

Table 2
Sensitivity of Detection of Ten ERβ Isoform Copy Numbers By Real-Time PCR with Reverse Transcribed cRNA

	Sensitivity (copies detected) at	
Isoform	50% Ramp Rate	25% Ramp Rate
ERβ1 (Wild Type)	10^2	ND[a]
ERβ2	10^2	ND
ERβ4	10^2	ND
ERβ5	10^2	ND
ERβ Exon 2Δ	10^2	ND
ERβ Exon 3Δ	10^3	10^2
ERβ Exon 4Δ	10^2	ND
ERβ Exon 5Δ	10^3	10^3
ERβ Exon 6Δ	10^3	10^2
ERβ Exon 5–6Δ	10^3	10^3

[a]ND, not determined.

and cell line suggesting that ERα-negative tumors could have estrogen signaling through ERβ isoforms. If proven by analysis of a large number of ERα-negative breast tumors, it could lead to a new line of ERβ-targeted therapies for ERα-negative patients. The results presented in Table 4 also indicate that every estrogen-responsive tissue has a unique composition in terms of types and quantities of ERβ isoforms. Variations in the ERβ isoform composition together with ERα isoform composition in these tissues, in part, could be the basis for tissue specific response to estrogens and SERMs.

The results presented in this report also demonstrate the high sensitivity, specificity, and efficacy of the developed assays in detecting and quantifying 10 ERβ isoforms as separate gene populations in picogram to nanograms amounts of total RNA in various target tissues. These assays

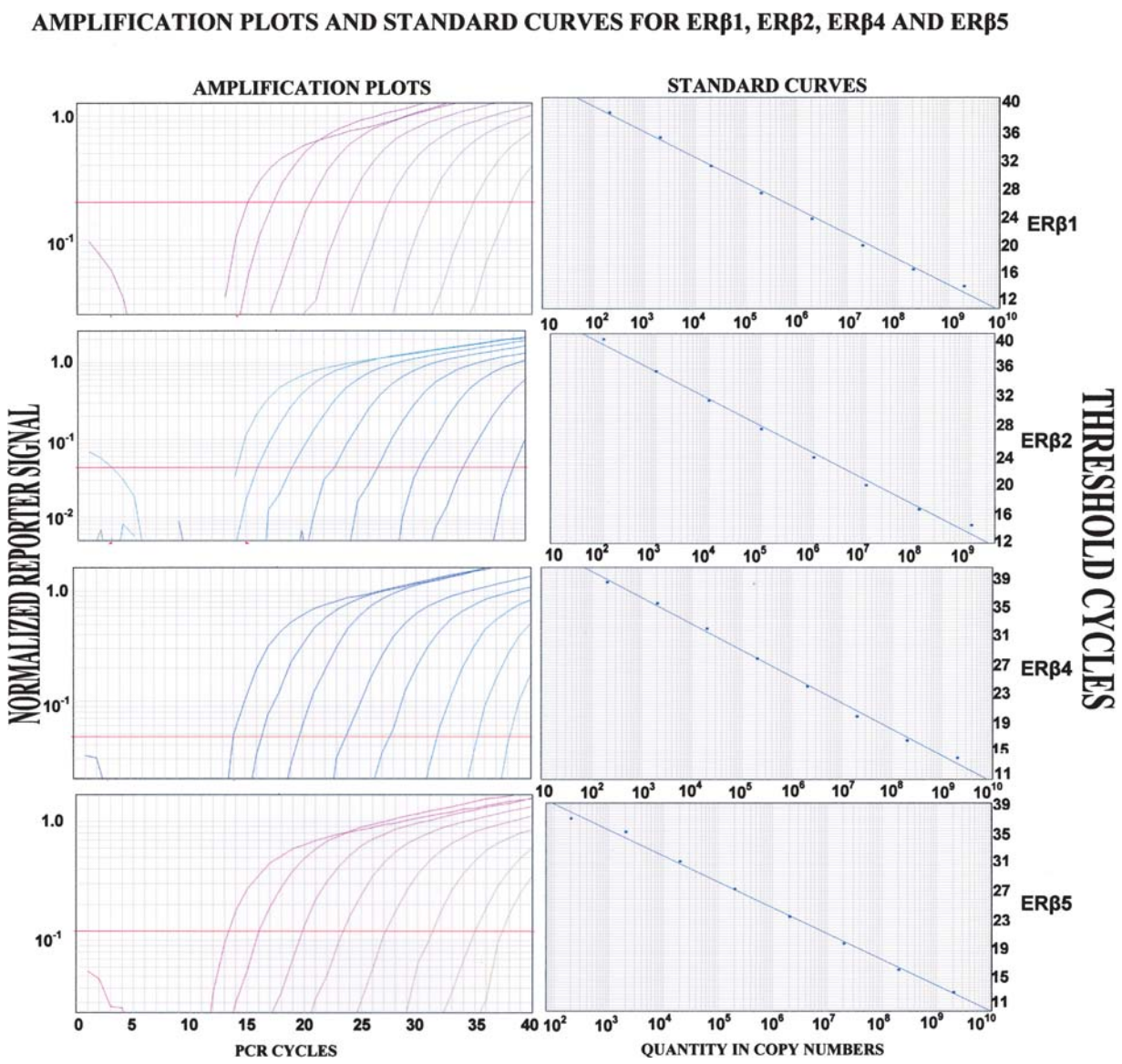

Fig. 4. Sensitivity of detection of ERβ1, ERβ2, ERβ4, and ERβ5 sequences with their respective reverse-transcribed cRNA. Left Panel. Amplification plots. Various copy numbers of reverse transcribed ERβ1, ERβ2, ERβ4, and ERβ5 cRNA were amplified by real-time PCR at 50% ramp rate in ABI Prism 7900 HT Sequence Detection System as described in *Methods* section. The amplification plots generated by ABI Prism 7900 HT [PCR cycle number (*X*-axis) vs normalized reporter signal (*Y*-axis)] are shown. The instrument normalizes the changes in the reporter dye (FAM) signals to a passive reference dye signal during the course of the PCR run and plots the graphs. The panels show the plots and standard curves for ERβ1, ERβ2, ERβ4, and ERβ5. The amplification plots shown from left to right in each panel were from 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 copies, respectively. Right Panel. Standard plots. The standard plots generated by the instrument for the copy numbers in A are shown. The quantities of the reverse-transcribed cRNA (*X*-axis) vs the threshold cycles (C_T, the first cycle at which an above background fluorescent signal is measured) (*Y*-axis) are plotted. The panels show the standard graphs generated for ERβ1, ERβ2, ERβ4, and ERβ5.

together with ERα isoform quantifying assays could be applied to profile and define the role of every ER isoform in estrogen-mediated signaling processes in a given tissue. It is also expected that profiling ERβ isoforms as well as ERα isoforms in different normal and pathological tissues may assist diagnostics and therapy of some diseases, as well as a search for factors causing this tissue-specific diversity and disease-related changes.

Materials and Methods

Omniscript reverse transcriptase kits were from QIAGEN Inc., Santa Clara, CA. All the primers used in the current study were synthesized by Gibco-BRL Life Technologies. TaqMan Universal PCR Master Mix (cat no. 4304437), RNAse inhibitor, and random hexamers were from Applied Biosystems. All the 5'FAM- and 3'TAMARA-labeled oligo-

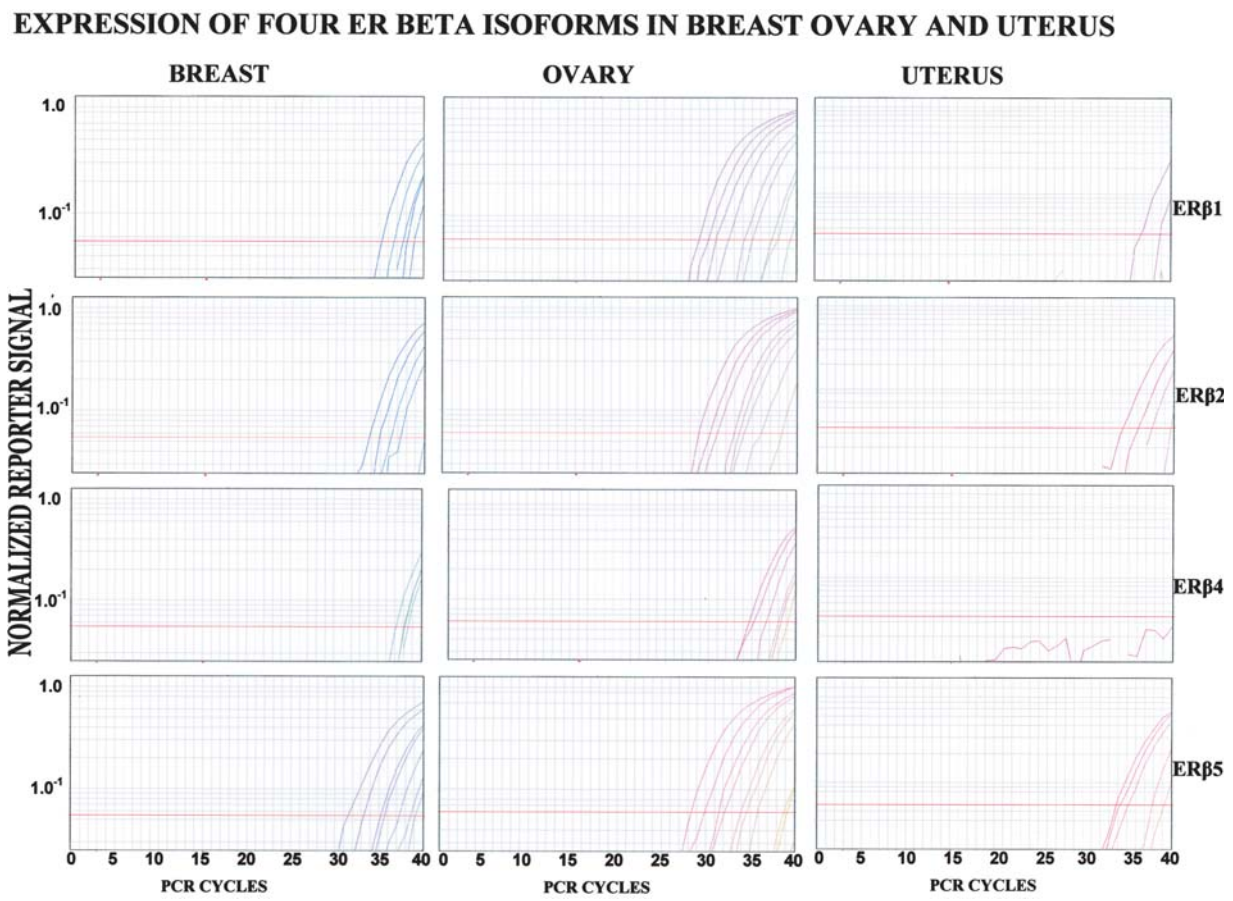

Fig. 5. Sensitivity of detection of ERβ1, ERβ2, ERβ4, and ERβ in reverse-transcribed total RNA from ovary, breast, and uterus. To determine the minimum amount of cDNA from ovary, breast, and uterus that could be detected for the above sequences, 50 ng of reverse-transcribed total RNA from each tissue was serially double diluted and amplified by real-time PCR at 50% ramp rate in ABI Prism 7900 HT Sequence Detection System as described in methods section. The amplification plots generated by ABI Prism 7900 HT [PCR cycle number (*X*-axis) vs normalized reporter signal (*Y*-axis)] are shown. The instrument normalizes the changes in the reporter dye (FAM) signals to a passive reference dye signal during the course of the PCR run and plots the graphs. The amplification plots obtained with primers and probes of ERβ1, ERβ2, ERβ4, and ERβ5 from each tissue are shown. Sensitivity of detection in breast. Amplification plots from left to right in ERβ1 panel were from dilutions, 1, 1:2, 1:4, 1:8, and 1:16, respectively; in ERβ2 panel, the plots from left to right were from dilutions 1, 1:2, 1:4, 1:8, and 1:16, respectively; in ERβ4 panel, the plots from left to right were from dilutions 1, 1:2, and 1:4, respectively; and in ERβ5 panel, the plots from left to right were from dilutions 1, 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64, respectively. Sensitivity of detection in ovary. Amplification plots from left to right in ERβ1 panel were from dilutions, 1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256, respectively; in ERβ2 panel, the plots from left to right were from dilutions 1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128, respectively; in ERβ4 panel, the plots from left to right were from dilutions 1, 1:2, 1:4, 1:8, 1:16, and 1:32, respectively; and in ERβ5 panel, the plots from left to right were from dilutions 1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256, respectively. Sensitivity of detection in uterus. Amplification plots from left to right in ERβ1 panel were from dilutions, 1 and 1:2, respectively; in panel ERβ2 panel, the plots from left to right were from dilutions 1, 1:2, 1:4, and 1:8, respectively; in ERβ4 panel, no plots were seen; and in ERβ5 panel, the plots from left to right were from dilutions 1, 1:2, 1:4, 1:8, and 1:16, respectively.

nucleotide probes described here were synthesized at Applied Biosystems. MEGAscript™ kit and DNAfree™ DNAse were from Ambion. PCR quality water and Tris-EDTA buffer were from BioWhittaker. Total RNA from breast cancer cell lines and tumors, human ovary, uterus and bone tissues were available from previous studies *(16,24–26)*. Normal breast tissue samples were collected from women undergoing reduction mammoplasty for cosmetic purposes at Howard University Hospital. Whole breast tissue obtained from reduction mammoplasty was used to isolate total RNA by Trizol method as described previously *(16,26)*.

ERβ1, ERβ2, ERβ4, and ERβ5 isoform coding sequences, 773 bp, 763 bp, 775 bp, and 745 bp, respectively, were amplified from human ovary cDNA using a sense primer in exon 4, 5'CGGCAAGGCCAAGAGAAG3' (position, exon 4, bp 824–841) *(27)*, and isoformspecific antisense primers in exon 8. The antisense primers for ERβ1, ERβ2, ERβ4, and ERβ5 were 5'AGCACGTGGGCATTCAGC3' [position exon 8, bp 1597–1580 *(27)*], 5'GTCACTGCTCCATCGT TGCT3' [position, exon 8, bp 1907–1887 *(20),* GenBank Accession No. AFO5 1428], 5'GTCTGGGTTTTATATCGT CTGCAA3' [position, bp 294–271 *(20)*, GenBank Accession

Table 3
Sensitivity of Detection of Ten ERβ Isoforms
in Human Breast, Ovary, and Uterus by Real-Time PCR

	Sensitivity of Detection (ng of reverse-transcribed total RNA)		
Isoform	Breast	Ovary	Uterus
ERβl	3.125	0.19	25
ERβ2	3.125	0.39	6.25
ERβ4	12.5	1.56	ND[a]
ERβ5	0.78	0.19	3.125
ERβ Exon2Δ	100	0.19	ND
ERβ Exon 3Δ	ND	100	ND
ERβ Exon 4Δ	12.5	0.39	6.25
ERβ Exon 5Δ	6.25	0.19	0.19
ERβ Exon 6Δ	6.25	0.19	6.25
ERβ Exon 5–6Δ	3.125	0.19	0.19

[a]ND, not detected.

Table 4
ERβ Isoform Copies/10^{10} Copies of GAPDH in Breast, Ovary, Uterus, and Bone Tissues by Real-Time PCR

Cell/ Tissue	ERβ1 Wild Type	ERβ2	ERβ4	ERβ5	ERβ exon 2Δ	ERβ exon 3Δ	ERβ exon 4Δ	ERβ exon 5Δ	ERβ exon 6Δ	ERβ exon 5–6Δ
Breast	5.8×10^4	6.2×10^4	3.3×10^5	7.0×10^6	3.3×10^3	0	1.1×10^6	2.3×10^6	4.0×10^4	6.2×10^6
Ovary	3.6×10^7	1.4×10^7	1.3×10^6	7.8×10^7	9.6×10^6	5×10^5	2.0×10^6	2×10^7	3.7×10^6	7.9×10^6
Uterus	1.4×10^5	4.0×10^4	0	3.2×10^7	3.5×10^4	0	1.2×10^6	1.5×10^7	3.5×10^4	8.5×10^7
Bone	0	1.1×10^3	0	1.7×10^5	0	0	0	0	0	7.6×10^3
MCF-7	8.5×10^4	1.1×10^5	0	2.8×10^5	4.2×10^3	0	3.5×10^6	5.7×10^5	0	5.7×10^4
MDA-MB-231	1.2×10^4	1.8×10^4	0	2.0×10^5	2.0×10^3	0	0	6.0×10^4	0	1.5×10^4
ERα + Breast Tumor	9.6×10^4	1.68×10^4	0	2.4×10^6	6.4×10^5	0	0	1.4×10^6	0	5.0×10^6
ERα – Breast Tumor	1.8×10^5	8.0×10^4	0	2.0×10^5	1.4×10^4	0	1.3×10^6	1.8×10^6	3.5×10^4	6.0×10^6

No. AFO6 1054], and 5'CACTTTTCCCAAATCACTTC ACC3' [position, bp 265–243 *(20)*, GenBank Accession No. AFO6 1055], respectively. The above products were cloned into pCR®2.1-TOPO plasmid and their identities confirmed by sequence analysis as described previously *(28)*. ERβ3 specific antisense primer, 5'CAGGCCTCAAAAGATCTGC TTC3' [position, exon 8, 1972–1993 *(20)*, GenBank Accession No. AF060555] was used to amplify its coding sequence, but it was found this isoform was not expressed in ovary, breast, uterus, or bone tissues confirming an earlier report *(20)*. ERβ exon 2Δ, 3Δ, 4Δ, 5Δ, 6Δ, and 5–6Δ splice variant coding sequences, 915 bp, 796 bp, 494 bp, 632 bp, 772 bp, and 633 bp, respectively, were cloned into pCR®2.1-TOPO plasmids and were available from previous studies *(24)*.

Preparation of Complementary RNAs (cRNAs) Using ERβ Isoform Coding Sequences Cloned into pCR®2.1-TOPO Plasmids as Templates

Absolute quantification of every ERβ isoform transcript copy number was achieved by comparison with a standard graph constructed using known copy numbers of cDNA of that particular ERβ isoform. Because reverse-transcribed tissue RNA is used for quantifications, the standard graph was also constructed with reverse-transcribed known copy numbers of cRNA. Complementary RNAs for eight ERβ isoforms were generated by in vitro transcription using plasmids containing their respective nucleotide sequences as templates. The cRNAs for each ERβ isoform were generated in the following steps: First, the pCR®2.1-TOPO vec-

tor containing the coding nucleotide sequences of ERβ isoforms were linearized by digesting with *BamH*I, extracted with phenol-chloroform, and precipitated with isopropanol. Second, the cRNA for each ERβ isoform was generated by in vitro transcription of 1–2 μg of the linearized plasmids using T7 RNA polymerase and other reagents supplied in the Ambion MEGAscript™ kit and following the manufacturer's protocol. Briefly, the transcription reaction mixture (20 μL) contained 1 μg of linearized ERβ isoform pCR®2.1-TOPO, 2 μL of 10X buffer, 2 μL each of 75 m*M* NTPs, and 2 μL of T7 RNA polymerase. The reaction mixture was incubated at 37ºC for 2–4 h. This generally produced 20–50 μg of cRNA. The cRNAs produced by this procedure were extracted with phenol-chloroform and precipitated with isopropanol to remove the T7 RNA polymerase and free unincorporated nucleotides and buffer salts. Third, the template plasmids from the in vitro transcribed cRNAs were removed by digestion with DNA-free™ DNAse. The added enzyme and the released nucleotides were removed by phenol-chloroform extraction, followed by precipitation with isopropanol. This procedure generally produced cRNAs devoid of any template plasmids as shown by conventional PCR in the absence of reverse transcription. The cRNA generated by the above procedure was quantified by measuring the optical density of the solution at 260 nm and the molarity was calculated based on the size (molecular weight) of the cRNA.

Reverse Transcription and Conventional PCR

The in vitro transcribed cRNAs and total RNAs from tissues/cell lines were reverse-transcribed using Omniscript reverse transcriptase as previously described *(16,24,25,29)*. Briefly, the cRNA/total RNA was denatured by heating for 3 min at 65ºC, cooled on ice, and incubated with reverse-transcriptase reaction mixture. The standard mixture contained 1 μg of total RNA, 10 U of RNAse inhibitor, 0.5 m*M* each of dNTPs, 1 μ*M* random hexamers, and 4 U of Omniscript reverse transcriptase in a total volume of 20 μL. For reverse transcription, tubes were incubated at 42ºC for 60 min, followed by 95ºC for 5 min, and finally rapidly cooled. Conventional polymerase chain reactions were performed in an automatic thermal cycler (MJ Research) as previously described *(30,31)* in a total volume of 12.5 μL containing the cDNA reverse transcribed from 125 ng of tissue total RNA/cRNA, 1X PCR buffer, 1X Q solution, 200 μ*M* each of dNTPs, 2 μ*M* each of sense and antisense primers, and 0.6 U of HotStartTaq polymerase. The PCR conditions were initial denaturation for 1 min at 95ºC, followed by 94ºC for 1 min, annealing for 1 min at the specified temperature depending on the primer pair used, extension for 2 min at 72ºC for 40 cycles, and final extension for 15 min at 72ºC. The annealing temperature for amplification of all the transcripts except exons 5–6Δ was 55ºC. For exons 5–6Δ, the annealing temperature was 61ºC *(24)*.

Primers and Probes for Quantification of ERβ1, ERβ2, ERβ4, and ERβ5 and ERβ Exon 2Δ, 3Δ, 4Δ, 5Δ, 6Δ, and 5–6Δ Isoforms by Real-Time PCR

The primer pairs and probes for the quantification of ERβ1, ERβ2, ERβ4, and ERβ5 and splice variant cDNAs by real-time PCR are listed in Table 1. The numbering of bp positions of ERβl and splice variants was based on cDNA sequence published by Ogawa and others *(27)*, and ERβ2, ERβ4, and ERβ5 was by Moore et al. *(20)*. The design, specificity, and applicability of splice targeted primers for the amplification of alternatively spliced ER sequences as separate gene populations have been established in a number of previous studies *(16,24,25,28,29,31,32)*. The specificity of each primer pair was verified by conventional PCR using their respective reverse-transcribed cRNAs and cDNA prepared from human ovary.

Absolute Quantification of 10 ERβ Isoform Transcript Copy Numbers by Real-Time PCR

Absolute quantification of ERβ isoform transcript copy numbers was achieved by quantitative real-time PCR in ABI Prism GeneAmp 7900HT Sequence Detection System at a modified 50% ramp rate or at 25% ramp rate as indicated. A typical real-time PCR reaction mixture contained cDNA prepared from reverse transcription of varying amounts of tissue/cell line total RNA/cRNA, 0.04 μ*M* each of sense and antisense primers, 0.05 μ*M* 5'FAM- and 3'TAMARA-labeled oligonucleotide probe, and 1X TaqMan Universal PCR Mix in a total volume of 25 μL. The PCR conditions were initial hold at 50°C for 2 min, followed by denaturation for 10 min at 95ºC, and denaturation for 15 s at 95ºC in the subsequent cycles and annealing and extension for 1.5 min at 60ºC for 40 cycles. An additional step of annealing at 55ºC for 15 s was added before the amplification step at 60ºC whenever the assay was conducted at 25% ramp rate. A standard graph for every ERβ isoform was simultaneously generated using 10^2, 10^3,10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 copies of its reverse-transcribed cRNA. The tissue/cell line cDNAs, reverse-transcribed cRNAs, primers, and probe were diluted on ice to the required concentration immediately prior to conducting PCRs. All the samples were amplified in triplicate and real-time PCRs were repeated four times for every isoform. GAPDH copy numbers were determined using a sense primer, 5'TTCCAGG AGCGAG ATCCCT3' (position, bp 304–322), an antisense primer, 5'GGCTGTTGTCATACTTCTCATGG3' (position, bp 483–505) and a probe, FAM 5'TGCTGGCGCTGAGTA CGTCGTG3' TAMARA (position, bp 342–363) in a given amount of cDNA.

Detection and Identification of PCR Products

The ERβ products (3.0 μL) generated by conventional PCR procedures were separated by electrophoresis in 1% Nu Sieve agarose gels in Tris-acetic acid- EDTA buffer and

detected by ethidium bromide staining. PCR products were purified by gel extraction, cloned into pCR®2.1-TOPO vector, and identified by sequence analysis as described previously *(28)*.

Acknowledgments

This work was supported by grants from the National Cancer Institute, National Institutes of Health (R33 CA88347) under Innovative Molecular Analysis Technologies (IMAT) program, the Department of Defense (Idea award DAMD 17-02-1-0409), and the Susan G. Komen Breast Cancer Foundation (BCTR0100473) to the author.

Abbreviations

FAM, carboxy-fluorescein; TAMARA, 6-carboxy tetraethyl-rhodamine; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; ERα, estrogen receptor alpha; exon Δ, exon deletion; ERβ, estrogen receptor beta, SERMs, selective estrogen receptor modulators.

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