CHAPTER THREE

MATERIALS AND METHODS

3.1

Sources of Tissue and Cell Lines

Samples were taken from 61 patients presenting at the Charlotte Maxeke Academic Hospital in Johannesburg with primary breast cancer. The breast fine needle aspirates (FNAs) were taken from patients by routine clinical procedure and placed immediately into lysis buffer containing guanidinium thiocyanate and B-mercaptoethanol (as provided by the RNeasy mini kit from Qiagen, Germany) and stored at -70°C. An aliquot of each FNA was also sent for routine cytology to verify the presence of tumour cells within each sample. Normal breast tissue samples were obtained from 10 patients undergoing breast reduction mammoplasty and stored in the same way. Ethical approval was obtained and all patients gave informed consent (Appendix I).

The MCF-7 breast carcinoma cell line from the American Type Tissue Culture Collection (ATCC HTB-22) was used as an ERα positive control and was maintained in Earle's Minimal Essential Medium (EMEM) (Gibco BRL, Life Technologies, UK) with 10% foetal calf serum (FCS) (Delta Bioproducts, Kempton Park, South Africa). The T-47D breast carcinoma cell line (ATCC HTB –133) was maintained in RPMI-1640 medium (Gibco BRL, Life Technologies, UK) with 10%

FCS supplemented with 0.2 I.U insulin/ml (10μg/ml Humulin N, Eli Lilly, Isando, South Africa) and 1mM Sodium Pyruvate (Gibco BRL).

3.2

Ribonucleic acid extraction

3.2.1

Human breast tissue

Ribonucleic acid (RNA) was extracted from human breast tissue using the Qiagen Rneasy Mini Kit (Qiagen, Germany). The samples were collected in a lysis buffer containing guanidinium thiocyanate and β-mercaptoetheanol as provided in the kit in order to protect the RNA. The tissue samples were manually disrupted using scalpel blades and the lysates were pipetted directly onto Qiashredder (Qiagen, Germany) spin columns and centrifuged in a microcentrifuge (Eppendorf centrifuge 5402, Eppendorf, Hamburg, Germany) for 2 minutes at maximum speed (14000 rpm). One volume of 70% ethanol was added to each homogenate and mixed well by pipetting to create conditions that promote selective binding of RNA to the RNeasy silica-gel membrane. Each sample was then applied to an RNeasy mini column (Qiagen, Germany), contaminants were washed away, and RNA was eluted from the columns in RNase-free water according to the manufacturer's instructions. The RNA was stored at -70°C until required.

3.2.2

Tissue culture cells

Medium was decanted from the flasks and the cells were washed with phosphate buffered saline (PBS) (Appendix II). 1ml lysis buffer containing guanidinium thiocyanate and β-mercaptoethanol (Buffer RLT, Qiagen, Germany) was added to each flask and the lysed cells were poured into Eppendorf tubes. The lysates were pipetted onto Qiashredder (Qiagen, Germany) spin columns and centifruged in a microcentrifuge for 2 minutes at maximum speed (14000 rpm). One volume of 70% ethanol was added to each homogenized lysate to adjust binding conditions. Each sample was applied to an RNeasy mini spin column, washed and RNA was eluted from the columns with RNase-free water according to the manufacturer's instructions. The RNA was stored at -70°C until required.

3.3

Reverse transcription

The extracted RNA was converted into complementary DNA (cDNA) prior to PCR to allow for the *Taq* DNA polymerase, used in the PCR, to function. The enzyme avian myeloblastosis virus (AMV) reverse transcriptase was used in this reaction.

A 20 μl reverse transcription reaction was performed for each FNA or tissue culture sample prior to PCR or first round nested PCR. 2 μl of a 10 x PCR buffer [500 mM KCl; 100 mM Tris-HCl (pH 8.4); 0.1% gelatin] was added to 4 μl $MgCl_2$

(25 mM), 2 μl (10 mM) dNTP mix (ie. 10 mM of each deoxynucleotide triphosphate (Promega, Madison, USA) was made up in a stock solution), 1 μl (20 U/μl) RNasin (Promega, USA), 2 μl oligo$(dT)_{15}$ (25 pmol/μl), 3 μl DEPC-treated water and 1μl (15 U/μl) of AMV reverse transcriptase (Promega). As the FNA samples were small and thus the concentration of RNA extracted was low it was not possible to quantitate the RNA spectrophotometrically for fear of losing all the RNA present. A random amount of 5 μl of RNA was therefore added to the reverse transcription reaction and mixed well. Initially, the RNA samples were incubated with the oligo$(dT)_{15}$ primer at 70°C for 5 min and cooled slowly to room temperature to facilitate primer annealing. After the addition of the remaining components the reactions were incubated at 37°C for 1 hour. The reverse transcriptase enzyme was inactivated by heating to 95°C for 5 min and cooling on ice. The cDNA was allowed to cool for 1 hour before amplification by PCR was carried out.

A reverse transcription control reaction was performed simultaneously by the addition of 5 μl DEPC-treated water in place of RNA in the reaction prior to reverse transcribing in all cases.

3.4 Polymerase chain reaction

The Polymerase Chain Reaction (PCR) is extremely sensitive and therefore precautions were taken to avoid contamination of extraneous DNA. Preparation of PCR reagents and amplification of samples were assigned to separate bench

areas. Pipettes were designated for PCR preparation, separated from others for general use and never used for pipetting PCR product or any potentially contaminating DNA source. Surfaces and pipettes were regularly wiped down with 1 M HCl and gloves were changed frequently. All primers used were synthesized by Invitrogen Life Technologies (Paisley, UK).

3.4.1

PCR of HPRT "housekeeping" gene

Hypoxanthine phosphoribosyltransferase (HPRT) is found in all cells as a soluble, cytoplasmic enzyme that catalyses the conversion of preformed bases guanine and hypoxanthine to guanylic and inosinic acids in the presence of phosphoribosyl pyrophosphate (PRPP) (Kim *et al*, 1986). The promoter of this gene resembles those of other characterized "housekeeping" genes and the functional HPRT gene is constitutively expressed (Patel *et al*, 1986). The entire gene is 42 kilobases (kb) in length and is organized into 9 exons and 8 introns (Kim *et al*, 1986).

In order to assess the integrity of the RNA extracted from the human breast tissue and the tissue culture cells each sample was reverse transcribed and amplified for the HPRT housekeeping gene. Samples positive for HPRT were analysed further for ERα and ERβ mutations.

3.4.1.1

HPRT primers

The forward primer HPRT-3, 5'-CCACGAAGTGTTGGATATAAGC–3', located in exon 7, begins at map position 604 of the HPRT gene (Jolly *et al*, 1983). The reverse primer HPRT-4, 5'-AGTTCAATGTTTCACTCAATAGTGC–3', begins at map position 1043 on the reverse cDNA strand and is located in exon 9.

3.4.1.2

HPRT PCR amplification conditions

20μl of cDNA product was amplified in a 50μl reaction volume containing 1 Unit of *Taq* polymerase (Gibco BRL, Life Technologies, UK); 1μM primer HPRT-3; 1μM primer HPRT-4; 5μl of 10 x PCR buffer (200mM Tris-HCl [pH 8.4]; 500mM KCl); and 1.5mM final concentration of $MgCl_2$. No additional dNTPs were added and the reaction was performed for 30 cycles in a programmable DNA Thermal Cycler (Hybaid, Middlesex, UK). After an initial heat denaturation at 96°C for 10 min, a program of 30 cycles of 94°C for 1 min, 56°C for 30 seconds, 72°C for 1 min 30 seconds and a final extension period of 10 min at 72°C was performed.

3.4.1.3

Visualization of HPRT PCR product

The PCR product was visualized by running on a 2% agarose gel containing 2μl ethidium bromide (10mg/ml) in TAE buffer pH 8.0 (Appendix II) at 80 Volts for 1 hour. A 50bp molecular weight ladder (Promega, Madison, USA) was run simultaneously and the gel was viewed on an ultra-violet light box and photographed. The HPRT PCR product was viewed as a band of 440 base pairs (Figure 16).

3.4.2

Nested and Touchdown PCR

In situations where there are problems with the quantity and quality of the template to be amplified one approach to amplifying a product reliably is to perform nested PCR. Generally, the sample is first amplified for 20-30 cycles using an outer primer set. A very small aliquot of this reaction is then amplified a second time using an inner primer set. The inner set of PCR primers are situated within the DNA so that the complementary sequence for the inner primer pair is present in the PCR product obtained in the first PCR reaction.

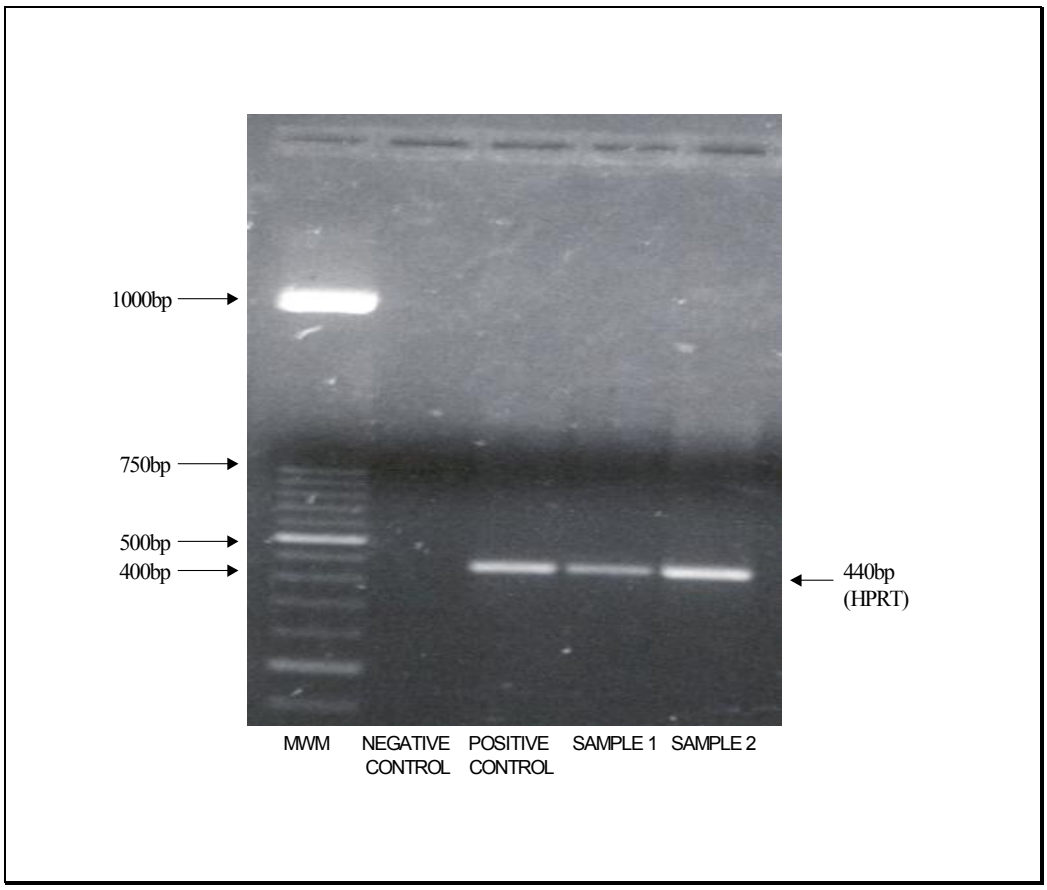

Figure 16. Visualization of HPRT PCR products on an agarose gel.

50bp ladder (Promega, SA) (lane 1); negative control (lane 2);
HPRT 440bp PCR product (lanes 3 to 5).

Nested PCR is extremely sensitive and as the clinical samples taken were very small, and thus the RNA obtained from them also minimal, nested PCRs for both ERα and ERβ were designed in order to be able to PCR the various exons using less material.

Artefacts may occur where incompletely extended products can occasionally rehybridize to an adjacent segment of DNA to prime an unintended product. The sequence internal to one or both primers will still be present, but the amplicon size will differ. The appearance of spurious smaller bands in the product spectrum due to mispriming by one or both of the oligonucleotide amplimers frequently dominate the reaction products and may be due to an advantage that the shorter misprimed products enjoy over the longer correct product during reaction cycling. One solution, which takes advantage of the exponential nature of PCR reactions, is "touchdown" PCR. Using this technique the annealing temperature of the reaction is decreased 1-2°C every second cycle from 65°C to a "touchdown" at a relevant annealing temperature. Any difference in T_m between the correct and incorrect annealing will give an advantage of 2-fold per cycle to the correct product (Don *et al*, 1991).

3.4.2.1

First Round Nested PCR of the ERα gene

The first round of nested PCR of the ERα gene was divided into two areas, namely, ERα DNA binding domain (ERαDBD) and the ERα ligand binding domain (ERαLBD). ERαDBD incorporated exons 1 to 6 and ERαLBD region incorporated

exons 3 to 8 (Figure 17). In ERαDBD Touchdown PCR an Enhancer solution (Gibco BRL, Life Technologies, UK) was used to facilitate efficient amplification of the problematic template.

PCR parameters for first round nested PCR of the ERα gene are reflected in Table 1.

3.4.2.1.1

ERα DNA Binding Domain PCR

The sense ERαDBD primer, ERA173 (5'–GGTTTCTGAGCCTTCTGC–3'), corresponds to bases 173-190 in exon 1 of the ERα gene (Green *et al*, 1986). ERA1484R (5'-CACATTTTCCCTGGTTCC–3') represents the anti-sense strand of the ERα cDNA bases 1467 -1484 in exon 6 of the ERα gene (Figure 17).

20μl of cDNA was amplified in a 50μl reaction volume containing 1μM primer ERA173; 1μM primer ERA1484R; 3.5μl of 10 x PCR buffer (200mM Tris-HCl [pH 8.4]; 500mM KCl); 0.78mM $MgCl_2$ and 5μl 10 x PCR Enhancer Solution (GIBCO BRL). No additional dNTPs were added and touchdown PCR was performed in a programmable DNA Thermal Cycler (Hybaid, Middlesex, UK).

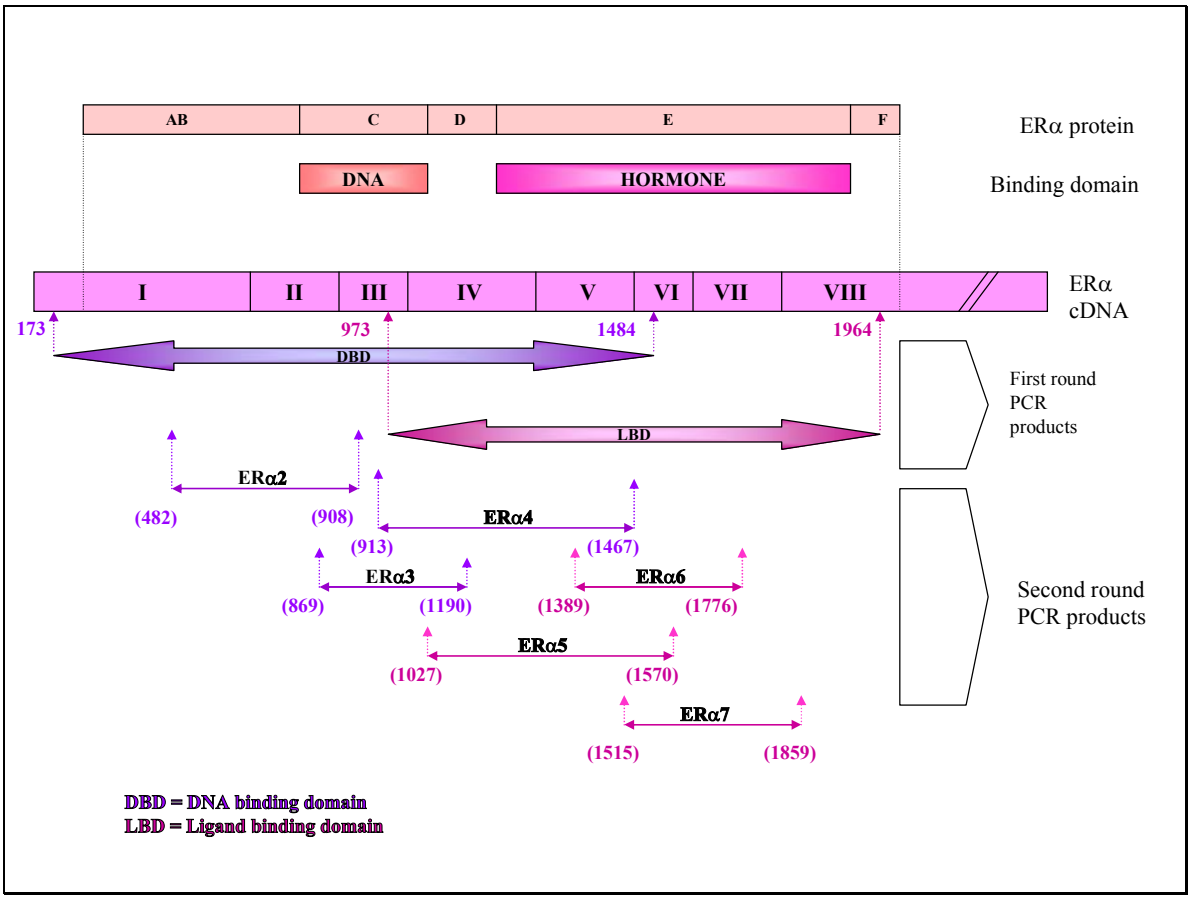

Figure 17. Positions of ERα nested PCR primers

(according to Green *et al*, 1986).

After an initial heat denaturation at 96°C for 10 min and an annealing temperature of 65°C for 1 min 1 Unit of *Taq* polymerase (Gibco BRL, Life Technologies, UK) was added and the reaction allowed to extend at 72°C for 2 min. Cycles consisting of denaturation at 94°C for 1 min, annealing for 1 min (where the annealing temperature was decreased by 2°C every 2 cycles starting from 65°C) and extension at 72°C for 2 min were carried out until the annealing temperature reached 57°C. A further 17 cycles were carried out with an annealing temperature of 55°C and a final extension period of 10 min at 72°C was performed.

The resulting PCR product of 1312bp was then used in second round nested PCR of exons ERα2, ERα3 and ERα4 as in 3.4.2.2.1. to 3.4.2.2.3.

3.4.2.1.2

ERα Ligand Binding Domain PCR

The sense ERαLBD primer, ERA973 (5'-AGTGGGAATGATGAAAGG-3'), corresponds to bases 973-990 in exon 3 of the ERα gene (Figure 17). ERA1964R (5'-AATGCGATGAAGTAGAGC-3') represents the anti-sense strand of the ERα cDNA bases 1947-1964 in exon 8 of the ERα gene (Figure 17).

20μl of cDNA was amplified in a 50μl reaction volume containing 1μM each of primer ERA973 and primer ERα1964R and 3.5μl of 10 x PCR buffer (200mM Tris-HCl [pH 8.4]; 500 mM KCl). No additional $MgCl_2$ or dNTPs were added and the reaction was performed in a programmable DNA Thermal Cycler (Hybaid,

Middlesex, UK). After an initial heat denaturation at 96°C for 10 min the temperature was reduced to an annealing temperature of 47°C for 1min before the addition of 1 Unit *Taq* polymerase (Gibco BRL, UK). An extension time of 2min at 72°C was performed and reaction was allowed to run for 30 cycles of 94°C for 1min, 47°C for 1 min and 72°C for 2 min. A final extension of 72°C for 5 min was then performed.

The resulting 922bp PCR product was used in second round ERα nested PCR of exons ERα5, ERα6 and ERα7 as in 3.4.2.2.4. to 3.4.2.2.6.

3.4.2.2.

Second Round Nested PCR of the ERα gene

ERα exons 2, 3 and 4 utilized the first round DBD PCR product as the template for second round PCR (Table 2). The first round LBD PCR product served as the template for second round PCR of ERα exons 5, 6 and 7 (Table 3 ; Figure 17). All second round PCR products were visualized as described in 3.5. The bands viewed were excised and purified using the phenol-freeze method (3.6) prior to quantitation (3.7) and sequencing in order to confirm the nature of the products observed (3.8).

Table 1. PCR parameters for first round ERα RT-PCR analysis.

Primer Name	Primer seq. 5'>>3'	Primer position	$MgCl_2$ conc.* (mM)	Ann. Temp. (°C)	ERα region detected	Product size (wt) (bp)
ERA173	GGTTTCTGAG -CCTTCTGC	173-190	0.78	TD 65-55	ERαDBD	1312
ERA1484R	CACATTTTCC -CTGGTTCC	1467-84				
ERA973	AGTGGGAATG -ATGAAAGG	973-990	-	47	ERαLBD	922
ERA1964R	AATGCGATGA -AGTAGAGC	1947-64				

TD = Touchdown

* $MgCl_2$ concentration in addition to that already present from the RT reaction.

In each case, if the original sample size was too small and no second round PCR product was observed after electrophoresis, the second round of PCR was repeated as described replacing the first round product with 2µl of second round product.

3.4.2.2.1

PCR of ERα exon 2

The sense ERα2 primer, ERA482 (5'-TCTGAGGCTGCGGCGTT–3'), corresponds to bases 482-498 in exon 1 of the ERα gene. ERA908R (5'-GGTTGGTGGCTGGACACA–3') represents the anti-sense strand of the ERα cDNA bases 891-908 in exon 3 of the ERα gene (Figure 17; Table 2).

5µl of first round ERαDBD PCR product was amplified in a 50µl reaction volume containing 1µM primer ERA482; 1µM primer ERA908R; 5µl of 10 x PCR buffer (Tris-HCL, KCL, $(NH_4)_2SO_4$, 15mM $MgCl_2$ [pH 8.7]; Qiagen, Germany); 200µM dNTP mix; 10µl of 5 x solution Q (Qiagen, Germany) and 1 Unit HotStar *Taq* DNA polymerase (Qiagen, Germany). The reaction was performed in a programmable DNA Thermal Cycler (Hybaid, Middlesex, UK) with an initial heat denaturation temperature of 95°C for 15 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. A final extension time of 10 min at 72°C was then performed.

The expected PCR product size was 427 base pairs (bp). An absence of exon 2 would reveal a PCR product of 236 bp.

3.4.2.2.2

PCR of ERα exon 3

The sense ERα3 primer, ERA869 (5'-ATTCAAGGACATAACGACT-3'), corresponds to bases 869-887 in exon 2 of the ERα gene. ERA1190R (5'-ACAAGGCACTGACCATCT-3') represents the anti-sense strand of the ERα cDNA bases 1173-1190 in exon 4 of the ERα gene (Figure 17; Table 2).

2μl of first round ERαDBD PCR product was amplified in a 50μl reaction volume containing 1μM primer ERA869; 1μM primer ERA1190R; 5μl of a 10 x PCR buffer (Qiagen, Germany); 0.5mM $MgCl_2$; 200μM dNTP mix; 10μl 5 x solution Q (Qiagen, Germany) and 1 Unit HotStar *Taq* DNA polymerase (Qiagen, Germany). The reaction was performed in a programmable DNA Thermal Cycler (Hybaid, Middlesex, UK) with an initial denaturation step of 95°C for 15 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 10 min was then performed.

The expected PCR product size was 322 bp. An absence of exon 3 (ERαΔ3) would produce a band 205 bp in size.

3.4.2.2.3

PCR of ERα exon 4

The sense ERα4 primer, ERA913 (5'-CACCATTGATAAAAACAGG-3'), corresponds to bases 913-931 in exon 3 of the ERα gene. ERA1467R (5'-CTGTCCAAGAGCAAGTTAG-3') represents the anti-sense strand of the ERα cDNA bases 1449-1467 in exon 5 of the ERα gene (Figure 17; Table 2).

2μl of first round ERαDBD PCR product was amplified in a 50μl reaction volume containing 1μM primer ERA913; 1μM primer ERA1467R; 5μl of a 10 x PCR buffer (Qiagen, Germany); 1mM $MgCl_2$; 200μM dNTP mix; 10μl 5 x solution Q (Qiagen, Germany) and 1 Unit HotStar *Taq* DNA polymerase (Qiagen, Germany). The reaction was performed in a programmable DNA Thermal Cycler (Hybaid, Middlesex, UK) with an initial denaturation step of 95°C for 15 min. This was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 1 min. The PCR program was completed with a final extension time of 10 min at 72°C.

The size of the expected PCR product was 555bp and a complete deletion of exonα4 (ERαΔ4) was expected at 219bp.

Table 2. PCR parameters for second round nested ERα DNA binding domain RT-PCR analysis.

Primer Name	Primer seq. 5'>>3'	Primer Position	Ann. Temp. (°C)	ERα region detected	Product size (wt) (bp)	Product size (variant) (bp)
ERA482	TCTGAGGCTG -CGGCGTT	482-498	55	ERα 2	427	236
ERA908R	GGTTGGTGGC -TGGACACA	891-908				
ERA869	ATTCAAGGAC -ATAACGACT	869-887	51	ERα 3	322	205
ERA1190R	ACAAGGCACT -GACCATCT	1173-1190				
ERA913	CACCATTGAT -AAAAACAGG	913-931	51	ERα 4	555	219
ERA1467R	CTGTCCAAGA -GCAAGTTAG	1449-1467				

3.4.2.2.4

PCR of ERα exon 5

The sense ERα5 primer, ERA1027 (5'-GAAACACAAGCGCCAGAGAG–3'), is located between bases 1027 and 1046 in exon 4 of the ERα gene. The reverse primer ERA1570R (5'-CACAAACTCCTCTCCCTGCA–3') corresponds to bases 1551 to 1570 in exon 6 on the anti-sense strand of the ERα gene (Figure 17; Table 3).

3μl of first round ERαLBD PCR product was amplified in a 50μl reaction volume containing 1μM primer ERA1027; 1μM primer ERA1570R; 5μl of 10 x PCR buffer (Qiagen, Germany); 1mM $MgCl_2$; 200μM dNTP mix; 10μl 5 x solution Q (Qiagen, Germany) and 1 Unit HotStar *Taq* DNA polymerase. The reaction was performed in a programmable DNA thermal Cycler (Hybaid, Middlesex, UK) with an initial denaturation at 95°C for 15 min. This was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min and extension at 72°C for 1 min. A final extension of 72°C for 10 min completed the program.

The ERα exon 5 PCR product size was 543 bp. ERαΔ5 would reveal a product of 405 bp.

3.4.2.2.5

PCR of ERα exon 6

The sense ERα6 primer, ERA1389 (5'-TCCTGATGATTGGTCTCGTCT–3'), is located between bases 1389 and 1409 in exon 5 of the ERα gene. ERA1776R (5'-CTGATGTGGGAGAGGATG–3') corresponds to bases 1759 to 1776 in exon 7 on the anti-sense strand of the ERα cDNA (Figure 17; Table 3).

3μl of first round ERαLBD PCR product was amplified in a 50μl reaction volume containing 1 μM primer ERA1389; 1μM primer ERA1776R; 5 μl of a 10 x PCR buffer (Qiagen, Germany); 0.5mM $MgCl_2$; 200μM dNTP mix; 10μl 5 x solution Q (Qiagen, Germany) and 1 Unit HotStar *Taq* DNA polymerase (Qiagen, Germany). The PCR was performed in a programmable DNA Thermal Cycler (Hybaid, Middlesex, UK) with an initial denaturation at 95°C for 15 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 1 min. A final extension time of 10 min at 72°C was subsequently performed.

The size of the expected PCR product was 388 bp. An exon 6 deletion would yield a PCR product of 254 bp.

3.4.2.2.6

PCR of ERα exon 7

The sense ERα7 primer, ERA1515 (5'-TGCTGGCTACATCATCTCG–3'), is located between bases 1515 to 1533 in exon 6 of the ERα gene. ERA1859R (5'-TCTCCAGCAGCAGGTCATA–3') corresponds to the cDNA bases 1841 to 1859 in exon 8 of the anti-sense strand of the ERα gene (Figure 17; Table 3).

3μl of first round ERαLBD PCR product was amplified in a 50μl reaction volume containing 1μM primer ERA1515; 1μM primer ERA1859R; 5μl of a 10 x PCR buffer (Qiagen, Germany); 0.5mM $MgCl_2$; 200μM dNTP mix; 10μl 5 x solution Q (Qiagen, Germany) and 1 Unit HotStar *Taq* DNA polymerase (Qiagen, Germany). The PCR reaction was performed on a programmable DNA Thermal Cycler (Hybaid, Middlesex, UK) with an initial denaturation time of 15 min at 95°C. This was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min. A final extension time of 10 min at 72°C was then performed.

The size of the expected PCR product was 345 bp. A complete exon 7 deletion, ERαΔ7, would yield a band 161 bp in size.

Table 3. PCR parameters for second round nested ERα Ligand binding domain RT-PCR analysis.

Primer Name	Primer seq. 5'>>3'	Primer Position	Ann. Temp. (°C)	ERα region detected	Product size (wt) (bp)	Product size (variant) (bp)
ERA1027	GAAACACAAGCGC-CAGAGAG	1027-1046	61	ERα 5	543	405
ERA1570R	CACAAACTCCTCTC-CCTGCA	1551-1570				
ERA1389	TCCTGATGATTGGT-CTCGTCT	1389-1409	59	ERα 6	388	254
ERA1776R	CTGATGTGGGAGA-GGATG	1759-1776				
ERA1515	TGCTGGCTACATC-ATCTCG	1515-1533	57	ERα 7	345	161
ERA1859R	TCTCCAGCAGCAG-GTCATA	1841-1859				

3.4.2.3

First Round Nested PCR of ERβ gene

The first round of nested PCR of the ERβ gene involved the PCR of a fragment of the ERβ gene located between exon 2 and exon 7. Great care was taken that the primers designed for this PCR in no way correlated with any sequence from the ERα gene. The first round PCR was designated $ER\beta_1$ (Table 4).

The sense $ER\beta_1$ primer, ERB528 (5'-AGGGATGCTCACTTCTGC–3'), is located between bases 528 and 544 (Ogawa *et al*, 1998a) in exon 2 (Enmark *et* al, 1997) of the ERβ gene (Figure 18). The anti-sense primer ERB1481R (5'-GAGCATCAGGAGGTTAGC–3') corresponds to bases 1464 to 1481 in exon 7 on the reverse strand of the ERβ gene.

20μl cDNA was amplified in a 50μl reaction volume containing 1μM primer ERB528; 1μM primer ERB1481R; 3.5μl of 10 x PCR buffer (200mM Tris-HCl [pH 8.4]; 500mM KCl) and 0.78mM $MgCl_2$. No additional dNTPs were added to the reaction. PCR was performed in a programmable DNA Thermal Cycler (Hybaid, Middlesex, UK). After an initial heat denaturation at 96°C for 10 min and an annealing temperature of 51°C for 1 min 1.5 Units of *Taq* DNA polymerase (Invitrogen, UK) were added and the reaction allowed to extend at 72°C for 2min. This was followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 1 min. A final extension time of 5 min at 72°C completed the reaction.

The resulting PCR product of 954 bp was used in second round nested PCR of the DNA and ligand binding domains of ERβ.

3.4.2.4

Second Round Nested PCR of ERβ gene

The second round of nested PCR of the ERβ gene utilized the first round PCR product as a template. One primer set was designed in order to PCR a segment of the ERβ DNA binding domain between bases 592 and 805 of the ERβ gene (ERβ exons 2 and 4 respectively) and one primer set was designed in order to PCR a segment of the ERβ ligand binding domain between bases 1196 and 1417 of the ERβ gene (ERβ exons 6 and 7 respectively) (Figure 18).

3.4.2.4.1

PCR of ERβ DNA binding domain

The sense ERβDBD primer, ERB592 (5'-CGTGTGAAGGATGTAAGG–3'), corresponds to bases 592 to 609 in exon 2 of the ERβ gene. ERB805R (5'-TCGGCACTTCTCTGTCTC–3') represents the anti-sense strand of the ERβ cDNA bases 788-805 in exon 4 of the ERβ gene (Figure 18).

Table 4. PCR parameters for first and second round ERβ RT-PCR analysis.

Primer Name	Primer seq. 5'>>3'	Primer Position	Ann. Temp. (°C)	ERα region detected	Product size (wt) (bp)
ERB528	AGGGATGCTCACT -TCTGC	528-544	51	$ER\beta_1$ First round	954
ERB1481R	GAGCATCAGGAGG -TTAGC	1464-1481			
ERB592	CGTGTGAAGGATG -TAAGG	592-609	52	ERβ DBD	214
ERB805R	TCGGCACTTCTCTG -TCTC	788-805			
ERB1196	GGGGAAATGCGTA -GAAGG	1196-1213	51	ERβ LBD	222
ERB1417R	ACCCAAACCAAAG -CATCG	1400-1417			

5μl of first round ERβ$_1$ PCR product was amplified in a 50μl reaction volume containing 1μM primer ERB592; 1μM primer ERB805R; 5μl of 10 x PCR buffer (200mM Tris-HCl [pH 8.4]; 500mM KCl); 1mM $MgCl_2$ and 200μM dNTP mix. The reaction was performed in a programmable DNA Thermal Cycler (Hybaid, Middlesex, UK). After an initial heat denaturation at 96°C for 10 min the temperature was reduced to an annealing temperarture of 52°C for 1 min before the addition of 1 Unit *Taq* polymerase (Invitrogen, UK). An extension time of 2 min at 72°C was performed and the reaction was allowed to run for 30 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min. A final extension of 72°C for 5 min was then performed.

As the ERβ signal was expected to be very low and due to the small sample sizes ERβDBD PCR was repeated as described replacing 5μl first round product with 2μl second round product. The final PCR product was run on a 2% agarose gel in 1 x TAE buffer as described in 3.5. The expected PCR product size was 214bp. The bands were excised, purified (3.6), quantitated (3.7) and sequenced (3.8).

3.4.2.4.2

PCR of ERβ Ligand binding domain

The sense ERβLBD primer, ERB1196 (5'-GGGGAAATGCGTAGAAGG–3'), corresponds to bases 1196 to 1213 in exon 6 of the ERβ gene. ERB1417R (5'-ACCCAAACCAAAGCATCG–3') represents bases 1400 to 1417 in exon 7 of the anti-sense strand of the ERβ gene (Figure 18).

5μl of first round ERβ$_1$ PCR product was amplified in a 50μl reaction volume containing 1μM primer ERB1196; 1μM primer ERB1417R; 5μl of 10 x PCR buffer (200mM Tris-HCl [pH 8.4]; 500mM KCl); 1mM $MgCl_2$ and 200μM dNTP mix. The reaction was performed in a programmable DNA Thermal Cycler (Hybaid, Middlesex, UK). After an initial denaturation at 96°C for 10 min the temperature was reduced to 51°C and 1 Unit of *Taq* DNA polymerase (Invitrogen, UK) was added to the mixture. After an extension time of 2 min at 72°C the reaction was run for 30 cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 1 min. A final extension of 72°C for 5 min was then performed.

Due to the small sample sizes and the low copy number of ERβ the ERβLBD PCR was repeated as described replacing 5μl first round product with 2μl second round PCR product. The expected size of the final PCR product was 222bp and this was visualized by running on a 2% agarose gel in 1 x TAE buffer as described (3.5). The bands were excised and purified (3.6) prior to quantitation (3.7) and sequencing (3.8).

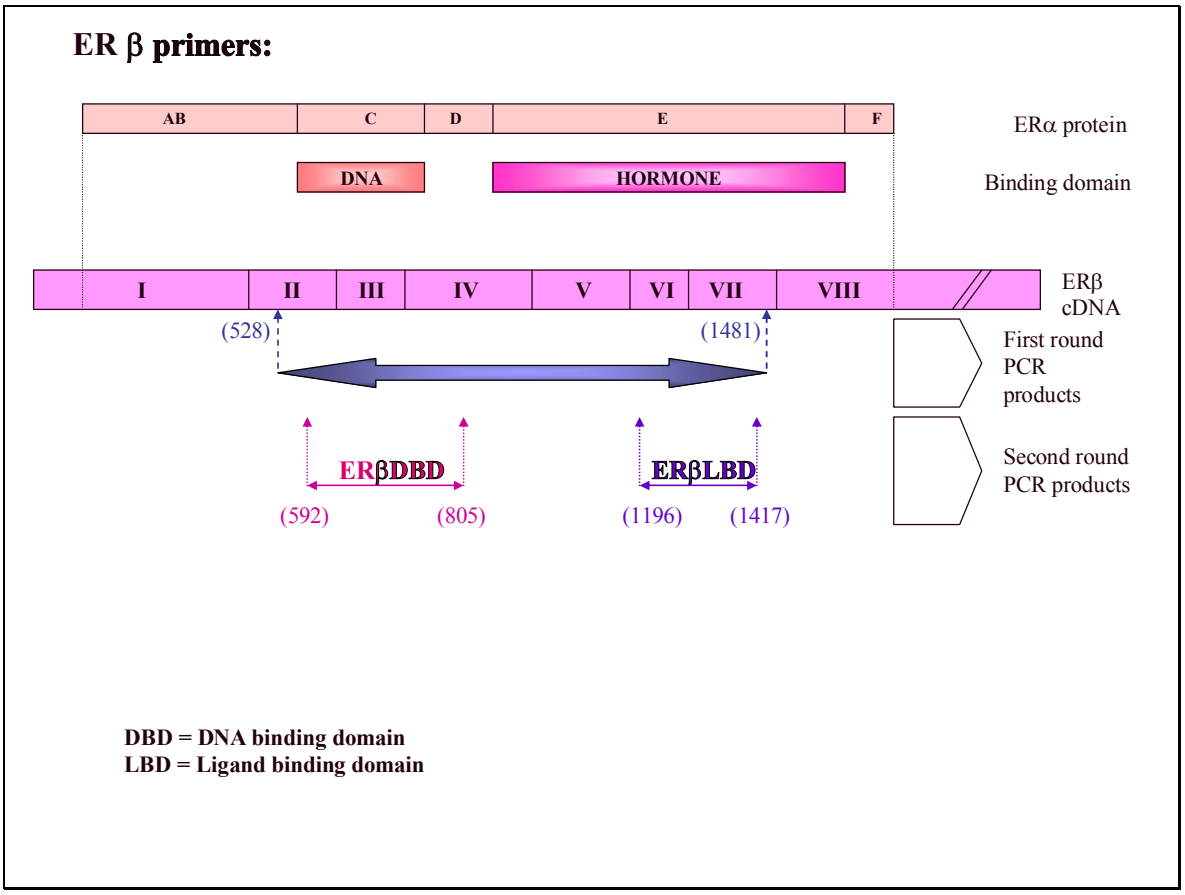

Figure 18. Positions of ERβ nested PCR primers

(according to Ogawa *et al*, 1998a and Enmark *et al*, 1997).

3.5

Agarose gel electrophoresis

Molecular Grade Agarose was obtained from Pronadisa (Madrid, Spain) and bromophenol blue and other chemicals from Merck (Darmstadt, Germany) unless otherwise stated.

In order to visualize the PCR products 2 μl loading buffer (5g sucrose; 50mM EDTA; 0.01g Bromophenol Blue; 1g Ficoll Type 400) was added 25μl of each PCR product and the PCR products were run on 2% agarose gels in 1 x TAE buffer (Appendix II) containing 1μg/ml ethidium bromide (Sigma, Germany) at 80V for 2 hours, viewed on a UV transilluminator (Spectroline, New York, USA) and photographed using a polaroid CU5 Land Camera with a 5-inch lens. Molecular weight markers run simultaneously were either the 100bp molecular weight ladder (Promega, Madison, USA) or MWM XIII (Roche, SA).

3.6

PCR product purification – Phenol-freeze method

The gel band was cut out of the gel and sliced finely with a sterile blade. The agarose slices were placed into an Eppendorf tube and 0.5 ml Tris-saturated phenol (ICN Biomedicals Inc., Ohio, USA) was added. The tube was vortexed until all the agarose was in solution (occasionally this also required heating at 50°C to assist the melting of the agarose). The agarose-phenol mixture was frozen rapidly

in an ethanol-dry ice bath for 2 min and centrifuged at 14000 rpm in a microfuge (Eppendorf, Hamburg, Germany) for 5 min. The freezing and centrifugation was repeated twice after which and aqueous phase was collected and transferred to a fresh Eppendorf tube. An equal volume of chloroform: isoamylalcohol (24:1) (Sigma Chemical Corporation, St Louis, USA) was added to the aqueous phase, mixed and centrifuged at 14000 rpm for 5 min. The resultant aqueous phase was transferred into a fresh Eppendorf tube and one tenth of the volume 3M sodium acetate [pH 5.2] (Appendix II) and 2 volumes of absolute ethanol were added and mixed. This mixture was stored at -20°C overnight to precipitate the cDNA. The mixture was centrifuged at 14000 rpm in a microfuge for 15 min at 4°C. The resulting precipitate was washed in 70% ethanol and recentrifuged for 15 min. The pellet was dried and resuspended in 12μl sterile water. The cDNA was quantitated as described in 3.7 prior to sequencing (3.8).

3.7

Ethidium Bromide Dot Quantitation of cDNA

This method was adapted from Wienand *et al* (1979).

DNA standards were prepared by diluting λ DNA (Roche Diagnostics, Randburg, South Africa) in sterile water at the following concentrations: 0ng/μl, 1ng/μl, 2ng/μl, 5ng/μl, 10ng/μl, 20ng/μl, 50ng/μl and 100ng/μl.

2μl of a 10ng/ml solution of ethidium bromide (Sigma, USA) was added to 2μl of each standard λ DNA solution and 2μl of each cDNA sample of unknown

concentration, respectively, and mixed well. The standard and sample DNA/ethidium bromide solutions were spotted onto plastic wrap placed on a UV transilluminator and photographed (Figure 19). The unknown cDNA concentrations were estimated by comparison to the fluorescence of the standards.

3.8

Sequencing of PCR products

50 to 100ng of each cDNA sample and 3.3pmol/μl of each relevant primer were provided to the Division of Human Genetics at the South African National Health Laboratory Service where the samples were sequenced with a Big Dye v3.1 Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in a programmable DNA Thermal Cycler with an initial denaturation step of 96°C for 1 min followed by 25 cycles of 96°C for 10 s, 51°C for 5 s and 60°C for 4 min with a final holding step of 4˚C. The sequencing reactions were performed in a 96 well plate and were subsequently cleaned by ethanol precipitation and the cleaned sequencing precipitates were resuspended in 8μl of loading buffer (5 parts deionized formamide to 1 part 25 mM EDTA [pH 8.0] with 50 mg/ml blue dextran). The samples were sealed, heated at 95˚C for 2 min and placed on ice prior to electrophoresis on an ABI377 automated sequencer (Applied Biosystems). Results were provided as spectrographs using Genescan®Analysis software (Appendices IV and V).

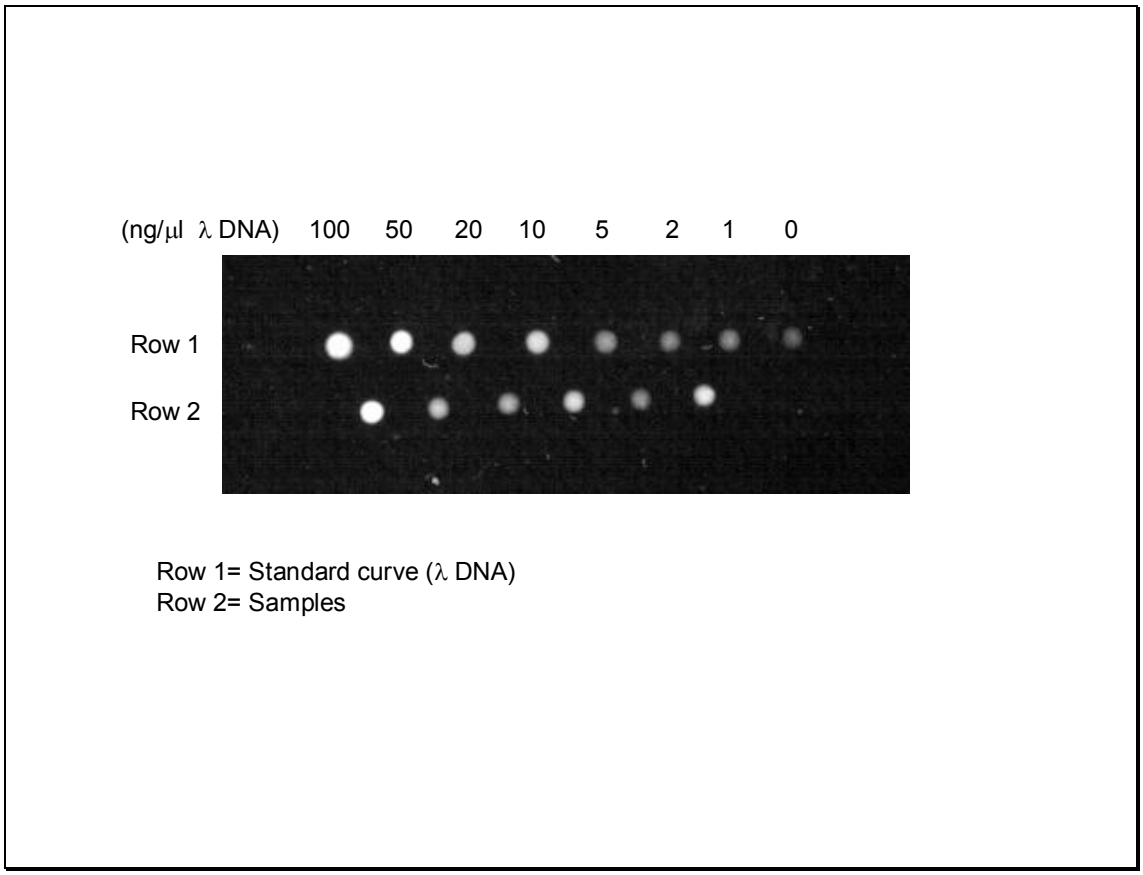

Figure 19. Ethidium Bromide dot quantitation of cleaned PCR products.

The concentrations (ng/μl) refer to the standard curve (Row 1).

3.9

Cloning of T-47D cell line

T-47D breast duct carcinoma cells were obtained from the American Tissue Culture Collection (ATCC HTB-133) and grown in Roswell Park Memorial Institute Medium 1640 (RPMI 1640, Gibco BRL, Life Technologies, UK) supplemented with 2mM L-glutamine, 0.2 I.U insulin/ml (10μg/ml Humulin N, Eli Lilly, Isando, South Africa), 1mM Sodium Pyruvate (Gibco BRL), 100 μg/ml penicillin/streptomycin mix (Gibco BRL) and 10% FCS. All media were phenol red free due to the oestrogenic effects of phenol red (Glover *et al*, 1988).

Clones of the T-47D line were derived by using a modified limiting dilution technique. Exponentially growing cultures were harvested by trypsinization, diluted, and plated into Nunclon (Kamstrup, DenmarK) 96-well tissue culture plates at 1 cell/ 100μl/ well. Each well was examined under phase contrast microscopy and any well not containing 1 cell was excluded. After 3 weeks of growth, 32 colonies were harvested and transferred into Nunclon 24-well tissue culture plates and after 12 weeks 13 clones were harvested and serially passaged to produce sufficient cells for cryopreservation of stock vials. After a further 12 weekly passages 5 clones were retained for further study, the remainder having been discarded on the basis of poor proliferation rates or bacterial or fungal contamination.

PCR for ERα and ERβ were performed as previously described and hormone response tests were performed on the 5 clones as described in 3.10. Based on the

results of these investigations two clones were selected for further study, namely, TCA3 and TCC1.

3.10

Hormone response test

Hormone response tests were performed every 24 hours (up to 96 hours) on both the parent clones and on each clone, 10 passages later, under control conditions and after exposure to 10^{-8}M 17-β-oestradiol, 10^{-8}M tamoxifen,10^{-8}M aminoglutethimide, 10^{-8}M androstenedione, 10^{-8}M estriol and 10^{-8}M cholesterol separately. 17-β- oestradiol and 4-hydroxy-tamoxifen were obtained from Sigma Chemical Co. (St Louis, MO, USA) and were dissolved in methanol. The final concentrations of methanol in the cultures were less than 0.1%.

Cells were harvested by trypsinization and washed in RPMI 1640 medium without phenol red or FCS. 1 x 10^4 cells were seeded into each well of a 96 well Nunclon plate containing 100μl indicator free RPMI 1640 medium and 10% stripped FCS (SFCS) (Appendix III) for each time frame (ie. 0h, 24h, 48h, 72h, 96h). 24 hours after plating (time 0h) medium was decanted from each well and 200μl fresh indicator free medium containing 10% SFCS was added to each well. Three control wells/clone/plate received only 0.1% of the vehicle methanol; 6 wells/clone/plate were supplemented with 10^{-6}M 4-hydroxy-tamoxifen (for 48 hours) after which 3 of these wells were replaced with medium supplemented with 10^{-8}M 17β-oestradiol for a further 48 hours; 6 wells were supplemented with 10^{-8}M

17β-oestradiol (for 48 hours) after which 3 of these wells were switched to medium supplemented with 10^{-6}M 4-hydroxy-tamoxifen for a further 48 hours.

At each time frame cell number was determined using the MTT assay as described in 3.10.1. A standard curve for each clone was run simultaneously and the MTT assay performed at time 0h.

3.10.1

MTT assay

This assay involves the ability of viable cells to convert 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT), a soluble tetrazolium salt, into an insoluble formazan precipitate (Slater *et al*, 1963) . The resulting purple-coloured formazan crystals are dissolved in an organic solvent such as dimethyl sulfoxide (DMSO) and the optical density of the solution may be measured on a multiwell spectrophotometer (ELISA plate reader) (Twentyman and Luscombe, 1987).

The tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) and the dimethyl sulfoxide (DMSO) were both obtained from Sigma (Germany). Optical density measurements were carried out using a Titertek Multiskan plate reader (ELISA reader) (Flow Laboratories, Rickmansworth, England) with a 540nm filter.

Cells were grown in 96-well plates (Nunc, Kamstrup, Denmark) in volumes of 200μl of medium per well as described previously in 3.10. The MTT was dissolved

in PBS (Appendix II) at a concentration of 5mg/ml and filtered through a 22μm filter (Millipore, Bedford, MA, USA). 20μl MTT was added to each well and incubated at 37°C in a CO_2 incubator for 4 hours. The medium was then carefully aspirated from the wells so as not to disturb the formazan crystals. 200μl DMSO was added to each well and the plates were agitated on a plate shaker for 5 min prior to reading the optical density at 540nm on a Titertek Multiskan plate reader. A standard curve was prepared for each cell clone and cell number was estimated as a comparison between the sample and standard optical density reading. In all cases 3 replicate wells were used to determine each point.

3.11

Staining of slides

Cells were grown on slides by plating into Nunc slide flasks containing 3ml of the relevant medium. The slide flasks were incubated in a CO_2 incubator at 37°C until cell growth was satisfactory. The slide flasks were removed from the incubator, the media discarded and the slides were gently prised off the flask. Slides were fixed by placing into absolute methanol for 5 min. Slides were stained in May and Grunwald's stain (BDH Chemicals Ltd, Poole, England) (Appendix III) for 5 min, in Giemsa stain (diluted 1:10) (BDH Chemicals Ltd) (Appendix III) for 10 min, rinsed in distilled water twice and mounted using solubilized glycerol jelly (BDH Chemicals Ltd). Slides were viewed at 40x magnification using the Zeiss Axioscop 2 MOT microscope (Carl Zeiss, Jena, Germany).