Quantitation of c-*myc* and Estrogen Receptor Gene Expression in Human Breast Cancer by Non-Isotopic Competitive RT-PCR

by

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QUANTITATION OF c-*myc* AND ESTROGEN RECEPTOR GENE EXPRESSION IN HUMAN BREAST CANCER BY NON-ISOTOPIC COMPETITIVE RT-PCR

BY

REKHA SINGH

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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Abstract

Initial success in the endocrine therapy of breast cancer is invariably followed by failure reflecting tumor progression and the emergence of estrogen independent growth. Manipulations in the expression of c-*myc* gene in cell lines has confirmed that c-*myc* is critical for the growth of breast cancer and is regulated by estrogen. Alteration of c-*myc* gene expression has been implicated in the cause and progression of breast cancer and the transition to hormone independent growth. To date no expression studies have taken into account the effect of time of tumor sample collection on the level of c-*myc* mRNA, given the potential for rapid decay rate of unstable c-*myc* gene mRNA which has a half life of 20-30 minutes in cultured cell lines. As a first step to determine the clinical relevance, we have studied the effect of specimen collection time of tumors on the level of c-*myc* expression. In this study, competitive reverse transcription polymerase chain reaction (RT-PCR) assays were developed using synthetic cRNA standards to quantitate c-*myc* mRNA and estrogen receptor (ER) mRNA extracted from small microdissected breast tumor samples.

The present c-*myc* assay could accurately distinguish a minimal 2-fold difference by comparison with northern blot and the ER assay could distinguish a 100 fold difference in ER level in mixtures of ER+ve and ER-ve RNAs. We then reassessed the relatively unstable c-*myc* mRNA and the stable ER mRNA in multiple homogeneous samples collected from breast tumor surgical specimens stored on ice for project times of 0, 3, 6 and 24 hours prior to freezing and subsequent RNA extraction. The levels of c-*myc* declined over 24 hours in each case (mean 74.7% of the level at time 0, sd 22.6 %, max 93%, min 31%, n=10) while ER levels showed a smaller change (mean 95% of the level at time 0, sd 3.2 %, max 98%, min 91%, n=4). The decrease in the rate of c-*myc* was independent of the tumor pathology assessed in the adjacent tissue blocks. This study suggests that the time after collection of surgical specimens may be an important parameter to assess the role of alteration of c-*myc* mRNA gene expression in human breast cancer.

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This thesis is dedicated to

my parents

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Abbreviations

A	adenosine
ADP	adenosine diphosphate
AMV	avian myelocytomatosis virus
ATP	adenosine triphosphate
AT	annealing temperature
AMV	avian myelocytomatosis virus
bp	base pairs
BR	basic region
BSA	bovine serum albumin
C	cytosine
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
cRNA	complementary ribonucleic acid
°C	degree Celsius
DBD	DNA binding domain
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DMBA	7, 12-dimethylbenzanthracene
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
DMSO	dimethylsulfoxide

ER	estrogen receptor
ER+ve/-ve	estrogen receptor positive/negative
ERE	estrogen receptor element
EGF	epidermal growth factor
FGF	fibroblast growth factor
G	guanosine
GTP	guanosine triphosphate
HBC	human breast cancer
H_2O	water
HBD	hormone binding domain
HCl	hydrochloric acid
HLH	helix-loop-helix
hsp	heat shock protein
IGF	insulin like growth factor
KCl	potassium chloride
kDa	kilodaltons
LOH	loss of heterozygosity
LTR	long terminal repeat
LFA	leukocyte function antigen
M/mM	molar/millimolar
μM	micro molar
μl	microliter
$MgCl_2$	magnesium chloride

MMTV	mouse mammary tumor virus
MMLV-RT	moloney murine leukemia virus-reverse transcriptase
MYC	c-*myc* protein
mRNA	messenger ribonucleic acid
Min/Max	minimum/maximum
NTP	nucleotide triphosphate
ng	nanogram
PAI	plasminogen activator inhibitor
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
pg	picogram
PR	progesterone receptor
RNA	ribonucleic acid
RNP	ribonuclear protein
RT	reverse transcription
RTase	reverse transcriptase
sd	standard deviation
SV40	simian virus 40
T	thymidine
Taq	thermus acquaticus DNA polymerase
TGF	transforming growth factor
Tris	tris (hydroxymethyl) amino methane
Zip	leucine zipper region

Chapter 1

Introduction

1.1 Breast Cancer-Pathology and Hormonal Influences:

Breast cancer is the most common malignancy among women in the western hemisphere and one of the leading cause of cancer-related deaths. Despite the identification of a number of biological and life style risk factors, no epidemiological risk-group can be discerned that has a greater than 30% life time risk of breast cancer (the sole exception being a small number of families with hereditary breast cancer) [1]. Even when the disease can be identified at a relatively early stage (i.e., node negative), 30% of women will still die of the disease [2]. Conventional pathological and clinical staging criteria do not allow an accurate assessment of the natural history of the disease or the impact of systemic adjuvant therapy in an individual patient. The identification of the specific molecular changes involved in breast carcinogenesis and progression will allow a more intelligent application of current therapeutic approaches and allow development of novel preventive and treatment strategies. One avenue is to explore the molecular mechanism of hormone action.

The potential importance of hormones in breast cancer was first shown in 1896 by the physician George Beatson when he observed temporary regression of the disease in his patients after removing the overies [3]. The naturally occurring estrogens are synthesized and secreted in large amounts by the theca cells of the ovarian follicles, by the corpus luteum and by the placenta, and in small amounts by the cells of the adrenal

cortex and by leydig cells of the testis. The observation that estrogens are the crucial mitogen in the development of breast tumors has been reaffirmed by numerous studies. The frequency of breast cancer in men and in women without functional overies is only 1% that of women with intact overies. Several risk factors for breast cancer in women are also closely associated with dose and time of exposure to estrogen. An early age at menarchy, late age at menopause and a late age at first full-term pregnancy result in prolonged exposure to elevated levels of estrogen and have been associated with an increased risk for breast cancer in women. Similarly obesity is also believed to increase the risk of breast cancer as elevated levels of estrogens result from aromatization of circulating androgens to estrogens in adipose tissue. In hormone responsive tumors significant growth arrest in response to endocrine therapy such as treatment with antiestrogen drugs like tamoxifen or surgical removal of the ovaries, adrenals and/or pituitary further supports the importance of estrogen in the development of breast cancer. Estrogen has been proposed to influence development of breast cancer through a variety of mechanisms such as: (1) Through regulation of several genes involved in DNA replication such as DNA polymerase [4], thymidine kinase [5], dihydrofolate reductase [6], thymidylate synthase [7], uridine kinase, carbamyl phosphate synthetase, aspartate transcarbamylase and glucose-6-phosphate dehydrogenase [7-9]: (2) Stimulation of the expression of a variety of growth factors such as insulin-like growth factors I and II (IGF-I and IGF-II) [10], and transforming growth factor α (TGFα) [8]. These growth factors in turn have been shown to be mitogenic for breast cancer cells [11]: (3) Transcriptional regulation of early response growth competence genes; c-*myc*, c-*fos* and c-*jun* [12-13].

Another potential mechanism of estrogen action leading to breast cancer

development has been proposed to involve hydroxylated intermediates of estradiol that have been shown to be carcinogenic. These hydroxylated intermediates occur at high levels in women with breast cancer or in women with a predisposition to the disease [14]. Other steroid hormones have also been implicated in breast cancer. Progestins, although growth stimulatory in DMBA-induced tumors in rats, are growth inhibitory for human breast cancer cells *in vitro* as are glucocorticoid, retinoids and vitamin D [15]. Androgens are growth inhibitory in some human breast cancer (HBC) cell lines in DMBA- treated rats; however, 85% of breast tumors contain androgen receptors and 7% of breast tumors are androgen growth dependent as is the mouse mammary tumor cell line S115 [16]. Thyroid hormones have been shown to be stimulatory in human breast cancer cell lines [17]. The mechanism beyond the receptors by which these hormones act on breast cancers is for the most part unknown. The inhibitory actions of progestins and androgens are at least in part due to a down-regulation of the estrogen receptor while retinoids have been described to down-regulate IGF-like binding proteins [18]. The peptide hormones prolactin, growth hormone and insulin have also been implicated in breast cancer. Studies have indicated that prolactin, whose receptor is present in 60% of HBCs, stimulates pathways associated with proliferation in breast cancer cells but no direct effect on growth rate itself has been observed. Human growth hormone has prolactin-like effects on breast cancer cells and is believed to act via cross-reactivity with the prolactin receptor [17]. Similarly insulin has been shown to be mitogenic for breast cancer cells and for DMBA-induced mammary tumors. In HBC cell lines insulin is believed to act by cross reactivity with the IGF-I receptor rather than through its own receptor [19]. In addition to hormones a variety of locally produced growth factors have also been implicated in breast

tumor growth. Included among these are TGF-α (transforming growth factor-α), EGF (epidermal growth factor), PDGF (platelet derived growth factor), FGF (fibroblast growth factor), IGF-I, IGF-II and TGF-β [20]. In addition to these established growth factors, two novel proteins, amphiregulin (an EGF- related protein) and mammastatin have also been characterized and shown to be growth inhibitory for several breast cancer cell lines [21].

1.2 Oncogenes and Human Breast Cancer:

The knowledge of specific genetic changes and their biological consequences is critical to an understanding of the natural history of breast tumors and the development of rational means to prevent and treat them. Oncogenes are perturbed normal cellular genes, which have been shown to confer properties to tumor cells or tumor-like cells essential for their behavior as tumor cells. Several of these genes have been shown to be evolutionarily conserved genes and to control important normal cell physiologic functions often related to cell growth and differentiation. When the normal control is disrupted by disregulation of the oncogene, this may contribute to tumor cell development. This disruption is due to alterations that either change the protein coded from the gene (point mutations) or the regulation of its expression (promoter insertion, amplification, translocation, regulation by virus). The oncogenes can be further grouped according to normal biochemical function or location. Several oncogenes are involved in growth factor regulation, either as growth factors, or receptors for growth factors. In several instances it seems that the oncogene is a mutated version of the growth factor receptor that may leave the receptor in a permanently activated state without any ligand/growth factor bound to it. Other oncogenes code for proteins involved in controlling the signal from the cell surface into the cell and to secondary messengers (G-like proteins, membrane bound

tyrosine kinases). Yet other oncogenes are active in the cytoplasm or show a nuclear location. The function of nuclear oncogenes is still obscure.

A number of oncogenes have been implicated in playing a role in breast cancer through mutation (truncation, amplification), or because they were originally found adjacent to the mouse mammary tumor virus (MMTV) integration sites in mouse breast cancer, and could possibly be under the control of the MMTV controlling promoter-enhancer element. These latter oncogenes *int-1* and *int-2* are named after virus *int*egration. These genes are frequently activated as a result of their location close to the strong MMTV-promoter-enhancer (the long terminal repeat, LTR). *int-1* is likely to code for a secreted protein, and thus is a candidate to be a growth factor which is highly conserved among diverse species, and in drosophila it is known to be involved in the development of wings and legs. *int-2* codes for a fibroblast growth factor (FGF) homologue. There are more members of this family discovered in the mouse system, but *int-1* and *int-2* are those known to be affected in some human breast cancers. *int-2* has been shown to be amplified in some human breast cancers [22].

c-*erbB-2/HER-2/neu* is closely related to the human EGF-receptor, but the ligand is unknown. *neu* is the rodent homologue of the human c-*erbB-2* (or *HER-2,* from human EGF-receptor 2). Putative ligands have recently been identified and it has been shown that if the intracellular part of c-*erbB-2* is linked to the extracellular domain of the EGF-receptor, the hybrid receptor can act as an EGF-receptor. This strongly supports the suggestion from the molecular homology to the EGF-receptor, that this is a membrane bound growth factor receptor. Amplification of this gene has been detected in up to 20% of stage 1 and 40% of stage II breast cancer patients, suggesting a role in progression of

the disease [23, 24].

The c-*myc*, a well studied nuclear oncogene (discussed in detail later) is amplified or over expressed in 22-32% of human breast cancer [25]. When introduced into mice as a transgene under the control of a strong-promoter-enhancer, c-*myc* can induce mammary cancers [26]. An extensive review on the role of c-*myc* in human breast cancer has been reported in [27]. A variety of other oncogenes including N-*ras*, K-*ras*, N-*myc*, L-*myc*, c-*myb*, v-*src* and c-*fos* have also been studied in breast tumors. Genetic mutations or aberrant expression of these oncogenes are more infrequent and as such their relevance to breast tumorigenesis remains uncertain.

The possible importance of the tumor suppressor genes in human breast cancer has been suggested by numerous studies that have identified alterations in both the p53 and Rb gene loci in these tumors [28-29]. Furthermore, the *BRCA1* and *BRCA2* genes have been shown to be altered in familial breast cancer [30-31]. The low production of protein encoded by *nm23* (metastasis suppressor) gene has also been correlated with reduced survival of breast cancer patients. Since suppressor gene mutations commonly involve total loss of one allele with a critical mutation or deletion in the other allele, loss of hetrozygosity (LOH) within the genome of tumor cells is believed to correspond to regions of the genome harbouring tumor suppressor genes. Analysis of breast tumor DNA for LOH has identified several frequently affected chromosomal regions including 1p, 1q, 3p, 11p, 13q14.1, 17p, 17q and 18q [32]. These results suggest the possible involvement of tumor suppressor genes in breast tumorigenesis and support the view that breast cancer develops as a result of multiple somatic mutations [33].

1.3 The c-*myc* Proto-oncogene:

The *myc* gene was first recognized as the principal oncogenic sequence of MC29, an acute retrovirus which causes sarcomas and carcinomas, as well myelocytomatosis and B-cell lymphomas, in chickens [34]. Although the precise physiological role of *myc* protein (*MYC*)is not known, there is overwhelming evidence suggesting its role in normal and abnormal cell proliferation [35].

Structural and functional characteristics:

The *myc* oncogene family consists of three well defined members c-, N-, and L-*myc* with several minor members R-, P-, and S-*myc* also described [36, 37]. The c-*myc*, N-*myc* and L-*myc* genes are distinct genes mapping to chromosomes 8, 2, and 1 respectively. They share a common organization of three exons and two introns with the major coding domain within the second and third exons (Figure 1.1). The c-*myc* gene, initially identified in humans has also been characterized in cat, mouse, rat, chicken, frog and in trout. The human c-*myc* gene is transcribed primarily from two promoters P1 and P2 [38]. P2 is within exon 1 and is 150 bp downstream of P1 (Figure 1.1). In all vertebrates studied, with the exception of c-*mycII* in *Xenopus*, P2 appears to be the principal promoter for transcriptional initiation. The minor Xenopus c-*myc*II transcript has an upstream start site designated as P' [39]. This transcript appears to resemble the 5% of c-*myc* transcripts seen in human B cell lymphomas that initiate from an upstream promoter P0, 550-650 bp from P1 [40]. For c-*myc* the polyadenylated mRNA produced from the P0, P1, and P2 are approximately 3.1, 2.4 and 2.2 kb respectively.

The human c-*myc* mRNA codes for two proteins, a 439 residue product initiating from the classical AUG codon in the second exon and a minor 455 amino acid protein

initiating from a CUG codon in the first exon [41]. Figure 1.2 shows the characteristic features of organization of the c-*myc* protein. The functional significance, if any, of the additional 16 amino acids in the larger protein is not known. Since the discovery of c-*myc* studies have been directed at identifying the biological function of this protein and subsequent family members. To date this function remains elusive; however, these studies have identified regions of *MYC* that could be important in its function such as the nuclear localization motif and helix-loop-helix/leucine zipper motif (HLH/Zip).

As mentioned earlier the *myc* proteins are nuclear proteins and as such require specific nuclear targeting sequences to facilitate their transport through the nuclear pore. This sequence has been identified in both the c-*myc* and N-*myc* proteins [42-43]. In the c-*myc* protein, the removal of residues 320-328 (PAAKRVKLD) resulted in its distribution in both the nucleus and cytoplasm [42]. Furthermore when these residues were fused to the pyruvate kinase protein, the fusion protein was directed into the nucleus [42]. These nuclear localization motifs are highly conserved and resemble the nuclear translocation signal in the SV40 large T antigen and steroid receptors described earlier.

The helix-loop-helix/ leucine zipper motifs are recognized as regions of structural similarity in a widespread group of transcriptional activators [44-45]. The basic DNA binding domain (DBD) in *myc* protein is followed by the helix-loop-helix (HLH) and the leucine zipper (Zip) motif. This peculiar dual motif arrangement has subsequently been recognized in a variety of transcription factors including TFE3, TFEB, USF and AP4 [46-49]. Deletion studies in the HLH-Zip region of USF and AP4 indicate that both regions are needed for dimer formation and DNA binding [48-49]. For c-*myc* and N-*myc*, mutation of either region can greatly impair or eliminate their co-transformation abilities,

their negative regulation of preadipocyte differentiation and their autonegative regulation [50]. Thus the carboxy-terminal 90 amino acids of *MYC*, containing the BR-HLH-Zip motif appears to be critical to its function and would suggest that the proteins forms dimeric complexes that may act as transcriptional regulators. By screening a cDNA library with a probe corresponding to the c-*myc* HLH-Zip region, a novel protein called *Max* was isolated [51]. This protein forms a heterodimeric complex with c-, N- and L-*myc* proteins, but not with other HLH or Zip containing proteins. The mouse equivalent of *Max* known as *Myn* has also been identified as a 160 amino acid, 18 kDa protein [52].

The *myc* proteins are all phosphoproteins. The significance of this phophorylation is unknown, however, deletion of phosphorylated regions affects the transformation phenotype of these proteins [53]. Non-specific and specific DNA binding has been associated with the *myc* proteins. Non-specific DNA binding has been proposed to participate in DNA replication [54]. The region of the c-*myc* protein involved in this DNA binding was localized to amino acids 265-318 and surprisingly did not involve the highly conserved basic region associated with the HLH-Zip motif [55]. Comparisons of c-*myc* proteins from other species indicate that residues 265-271 and 290-318 are highly conserved. Transformation studies using *myc* mutants lacking these regions yielded mixed results, with some mutants exhibiting normal co-transformation activities and others unable to transform [56]. Despite the inability of the HLH-Zip DNA binding basic region [BR] to bind the sequence used to identify the non-specific DNA binding site, domain swapping experiments in which the E12-basic DNA-binding region was replaced with the corresponding c-*myc* region, revealed binding of the E12-c-*myc*-BR homodimeric complex to a CA-TG consensus sequence [52]. Alternate approaches have

also recognized CACGTG as the *MYC-Max* heterodimer specific binding site [57]. This element coincides with part of the binding sites described for the human transcription factors USF and TFE3 [58]. The *MYC-Myn* complex in the mouse has been shown to bind this core sequence CCACGTGA preferentially when preceded by a GA or AG at the 5' end and followed by a TC or CT at the 3' end [52]. Therefore the BR associated with the HLH-Zip in the *myc* protein appears to bind a specific DNA element as do the corresponding regions of other HLH and/or Zip proteins. Methylation of this recognition site inhibits complex binding and as such methylation changes associated with growth, differentiation and transformation have been proposed as a possible regulatory mechanism of *MYC* action *in vivo* [52].

c-*myc* is an early response, cell cycle competence gene [59]. *MYC* can transform embryo fibroblasts when expressed together with the p21 c-*H-ras* protein [60] or when co-transfected with a variety of other oncogenes including c-*raf-1* and *bcl-2* [61-62]. For this reason *MYC* is suspected to play a major role in cellular proliferation and differentiation. The mechanisms by which *MYC* potentiates these functions are not very well understood. The observation that the *myc* proteins are the members of the helix-loop-helix (HLH) and leucine zipper (Zip) gene family suggest that *MYC* is a transcription factor. A small group of genes have been identified to be regulated both positively and negatively by *MYC*. Included in this group are the *hsp70* and E4 promoters, the plasminogen activator inhibitor-1 (PAI-1) and the ∝-prothymosin gene, all of which are activated by *MYC* [63-66]. Conversely the metallothionein promoter appears to be suppressed by the *myc* protein as are the leukocyte function antigen (LFA-1) receptor, the neural cell adhesion molecule (N-CAM), variant histones H1 and H1° and the major

histocompatibility complex (MHC) cell surface proteins [67-71]. The mechanisms of *myc* regulation of these genes are poorly understood and appear to vary considerably. In the case of the *hsp70*, E4 promoter and ∝-prothymosin, a direct effect on transcription appears probable, although there is no direct evidence of a *MYC* complex with these promoters. In the case of the LFA-1, a tetrameric protein, *MYC* is suspected of decreasing ∝1-chain mRNA expression via a post-transcriptional mechanism involving α1-chain RNA processing or transport [72]. Post-transcriptional mechanisms are also believed to be involved in the induction of PAI-1 [65]. Suppression of MHC cell surface proteins by *MYC* is thought to result from a post-translational modification of the H2K enhancer *trans*-activator H2TF1 [73].

In addition to regulating the aforementioned promoters, *MYC* has been shown to down-regulate its own expression [74]. Negative autoregulation of *myc* expression was first observed in Burkitt's lymphomas where translocation of the c-*myc* gene placed it under the regulation of the strong immunoglobulin regulatory region [75]. This enhanced, deregulated c-*myc* expression prevented c-*myc* transcription from the other, untranslocated c-*myc* allele. This autoregulation also appears most pronounced in non-transformed cells suggesting that transformed cells may have acquired a growth advantage through a disruption in this mechanism [76]. Although the mechanism of autoregulation is not known other trans-acting factors are believed to be involved. Mutational studies of the *myc* protein have revealed two regions that if mutated can interfere with the autoregulatory function. One of these region is BR-HLH-Zip region and the other region is a region spanning amino acids 105-143. Incidentally both these regions are also essential for co-transforming activity, indicating a link between transformation

and autoregulation.

The c-*myc* expression has been generally linked to the rate of cellular proliferation, both in *in vitro* and *in vivo* systems [77-78]. Quiescent cells in culture and nonreplicating somatic tissues have low levels of c-*myc* expression [79]. On the contrary, cells constitutively expressing a high level of c-*myc* show a shorter G1, require less growth factors, and do not arrest in G0 [80]. Thus c-*myc* expression is believed to drive at least one step of the cascade of events that follows mitogenic stimulation of quiescent cells to proceed into the S phase. Several lines of evidence have also suggested a different key role played by c-*myc* in the control of growth, differentiation, appoptosis and in DNA replication in various cell systems [81-84].

Regulation of c-*myc* expression:

In general terms c-*myc* mRNA accumulation is known to increase in mitogenically stimulated, actively proliferating cells, and to decrease in cells committed to terminal differentiation. Studies addressing these aspects of c-*myc* expression have revealed an array of regulatory mechanisms which can be divided into two groups: (1) transcriptional mechanisms involving either initiation or elongation/termination of new transcripts or (2) post-transcriptional mechanisms principally involving mRNA stability.

In the c-*myc* gene, several lines of evidence point towards the 5' flanking region, exon 1 and possibly intron 1 as the gene regulatory regions. A variety of growth factors and mitogens have been shown to stimulate c-*myc* gene expression by increasing initiation of new transcripts [85-86]. Regulatory elements are believed to influence both initiation [87, 88] and elongation [89] of transcription. Both enhancer and repressor regions have been identified within the *myc* regulatory region. Two enhancer regions, one

encompassing the PI & P2 promoters and the other region far upstream in the Dnase I hypersensitive site I between -2300 bp to -1980 relative to P1 [90]. In addition two repressor regions NRE1 and NRE2 encompassing hypersensitive regions II_1 and II_2 respectively have also been defined [90]. NRE1 mapped between -1527 bp to -1246 while the strong regulatory region NRE2 mapped between -1052 bp to -607 bp [89].

Other groups have also reported negative regulatory elements [91-94]. Most groups have extended this region to include sequences up to -428 bp [92-93]. More careful dissection of this region in mouse c-*myc* has revealed that the 5' and 3' ends possess the dehancer function [95]. In general reported enhancer and dehancer regions appear to overlap. In addition to identifying positive and negative regulatory regions, transfection studies have also identified minimal sequences necessary for P1 and P2 promoter usage. In human c-*myc* -60 bp to -37 relative to P1 are essential for its activity [96]. Similarly sequences upstream of P2 from -7 bp to 116 appear essential for P2 usage in both the mouse and human c-*myc* [85]. Within this region is a positive *cis*-acting element ME1a1 that if removed results in preferential usage of the weaker P1 promoter [85]. Within the positive and negative regulatory regions of the c-*myc* gene are a number of *cis*-acting elements, and in some cases the corresponding *trans*-acting factors binding these elements, have also been characterized. The *cis*-acting elements 5'Mf, 5'Mg1, 5'Mg2, 5'Mg3, ME1a1, ME1a2 were identified in mouse c-*myc* [85]. The corresponding human sequences are very similar particularly for ME1A1, ME1a2, 5'Mg3 and 5'Mf [85]. Near the core promoter region, a *cis*-acting DNA sequence element that binds a ribonucleoprotein (RNP) has been identified [97].

Several transcriptional repressors have also been identified with binding sites

overlapping activator binding sites [98]. Other ubiquitous transcription factors including NF-1, AP-1, E2F, AP-1 and octomer binding factor have all been shown to bind the c-*myc* gene and as such may be involved in the complex regulatory mechanism of this gene [99-102]. Although differentiating cells exhibit a decrease in c-*myc* initiation, the majority of transcripts initiate properly from the P1, P2 promoters but elongate poorly and prematurely, generating highly unstable, untranslatable RNA [103]. Transcriptional attenuation and premature termination are important regulatory mechanisms of many prokaryotic and eukaryotic genes. In the c-*myc* gene, multiple attenuation sites are observed with transcriptional termination occurring preferentially at two thymidine stretches, T1 and T2, near the exon1/intron1 boundary [104]. These T stretches are insufficient to block a heterologous gene, although a region encompassing T1, T2 and 125 bp upstream have been shown to regulate the attenuation processes [105].

The early response genes of which *myc* is a member, share common characteristics in that they all encode unstable mRNAs which are rapidly and transiently expressed upon mitogenic stimulation and are superinduced upon treatment with protein synthesis inhibitors such as cyclohexmide [105]. When stimulated by mitogens, expression of these early genes increase; however, in most systems, transcriptional initiation alone cannot account for the level of mRNA observed and therefore post-transcriptional mechanisms must also be involved. Under normal conditions the P1 and P2 derived mRNA are extremely unstable with half-lives of about 20 minutes [106-107]. The principal difference in the *myc* mRNAs are variable 3' untranslated regions of 350, 900, and 1900 bp in c-, N- and L-*myc* respectively. However, contained throughout this 3' untranslated region are multiple AU-rich regions that are believed to account for the short

half life of these mRNAs [108]. In some plasmacytomas and Burkitt's lymphomas, the deregulated c-*myc* mRNA is ten times more stable with a half life of several hours instead of 20-30 minutes [109-111]. As most of these translocated *myc* genes lack 5' flanking and exon1 sequences, it was hypothesized that these regions also contained sequences essential to regulating c-*myc* mRNA stability. However a subsequent study aimed at confirming this hypothesis found that removal of the 5' flanking and exon1 sequences had only a small effect on c-*myc* mRNA stability [112]. Conversely, removal of a 140 bp AU-rich region from the 3' untranslated end of the c-*myc* mRNA greatly increases *myc* mRNA stability. The destabilizing effects of the AU-rich motif may be due to its signaling a shorter polyA tail or to its recognition by specific endonuclease [113-114]. When the 3' ends of stable genes were replaced by the AU-rich region, the heterologous transcripts produced by these genes were unstable. Even so, the AU-rich region is not solely responsible for c-*myc* mRNA stability as some mutant *myc* constructs containing only a mutation in the 5' flanking region/exon1 and not in the AU motif also produce stable mRNA [112]. This result indicates that certain 5' sequences can overcome AU-motif destabilization in c-*myc*.

In addition to the AU-motifs, there is also evidence of an additional secondary destabilization mechanism coupled to translation [115]. The molecular determinants essential to this translational destabilization have been localized to the region of the mRNA coding for amino acids 335-439 [116]. This region can impart a translational destabilization signal to a heterologous gene. Whether the destabilization signal is present in the mRNA or the nascent c-*myc* peptide is unknown. It has been suggested that the helix-loop-helix region 360-396 may be interacting with another protein resulting in a

transduction signal for mRNA degradation [116]. Thus increased transcriptional initiation and post-transcriptional *myc* mRNA stabilization might be responsible for enhanced c-*myc* expression in mitogen stimulated cells resulting in disruption of normal growth and differentiation process resulting in tumor formation.

Deregulated expression of the c-*myc* gene:

The c-*myc* gene is regulated at multiple levels and alteration of expression might therefore occur, at least in theory by a number of mechanisms, and has been associated with many tumors. These deregulatory mechanisms can be separated into two groups: (1) Those affecting the physical gene structure, such as retroviral insertion or transduction, chromosomal translocation and gene amplification/rearrangement: and (2) Those affecting the expression of mature *myc* transcripts. Promoter insertion by integration of avian leukosis virus sequences adjacent to the c-*myc* gene in chickens [117] results in bursal lymphomatosis, a B-cell malignancy initiated by transcriptional activation of the host oncogene. Likewise, the reciprocal translocation of c-*myc* and immunoglobulin gene sequences characteristic of both murine plasmacytomas and human Burkitt's lymphomas [118] frequently results in inappropriate expression of the translocated c-*myc* allele. The c-*myc* gene amplification has also been reported in tissue culture cell lines derived from human promyelocytic leukemia [119], human colon cancer [120], human breast carcinoma [25] and human small cell lung carcinoma [121].

The role of c-*myc* in breast cancer:

The importance of c-*myc* gene in breast tumorigenesis was first demonstrated when transgenic mice over expressing *MYC*, under the transcriptional control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) enhancer/promoter,

developed mammary tumors after a relatively long latent period [26]. The importance of c-*myc* gene product in the regulation of cell proliferation is continually being reaffirmed by its involvement in mitogen-stimulated cell growth. The majority of these mitogens have been peptide growth factors such as PDGF, FGF, EGF and growth hormone that activate cell growth and c-*myc* induction through cell surface receptors coupled to intracellular signal molecules, such as calcium, protein kinase C, metabolites of phosphatidyl-inositol and cAMP. Unlike peptide growth factors, however, estrogen acts through a nuclear receptor which can bind DNA and activate specific genes. It has been shown that estradiol activates c-*myc* transcription within minutes and that this activation is not inhibited by cyclohexmide; also the estradiol does not alter the stability of c-*myc* mRNA [12]. An estrogen response element (ERE) has been identified within the c-*myc* regulatory region, which is believed to span some 2000 bp upstream of the first exon and possibly all or part of the first intron. Contained within the regulatory region are the three c-*myc* promoters, P0, P1and P2 of which P2 is the major start site in humans. Also identified in this region are numerous enhancer and dehancer subregions [88, 92, 93, 95], indicating that regulation of the c-*myc* gene expression is highly complex. However, the ERE of the human c-*myc* has been localized within a 116 bp sequence 5' of the P2 promoter [122]. This region lacks a conventional palindromic ERE sequence, suggesting a novel mechanism of estrogen *trans*-activation of the human c-*myc* gene.

In support of a role for c-*myc* in the growth of breast cancer, using c-*myc*-specific antisense oligonucleotides, it has been shown that the estrogen-associated proliferation of human breast cancer cells is critically dependent on the presence of the c-*myc* but not on the presence of the pS2 gene product [123]. In breast cancer cells that are ER+ve and

hormone dependent, c-*myc* is directly regulated by estrogen, [12, 122] whereas in contrast, the regulation of c-*myc* appears to be altered in ER-ve hormone independent cells where expression of c-*myc* may be high and constitutive [124]. The mechanisms that underlie this alteration in regulation are not always clear but it has been shown that in estrogen responsive breast cancer cells (MCF7), increased expression occurs as a result of enhanced transcription, while in estrogen independent breast cancer cells (MDA-MB-231), post-transcriptional mechanisms, whether it be enhanced processing, transportation, or mRNA stabilization appear to play a major role [12, 124-125].

Although c-*myc* is shown to be a primary target in the mechanism of estrogen action, its expression can also be influenced by progestins and retinoic acid [126-127]. At the same time all three steroid hormones have been shown to modulate invasiveness in estrogen receptor positive breast cancer cells [128]. While these hormonal effects might well be the result of direct effects of each steroid on genes regulating collagenase and the laminin receptor [129], it is possible that the effects of several steroid hormones (estrogen, progesterone, reteinoic acids, etc.) on invasion are partly mediated through c-*myc* and its downstream genes. Among candidates for *myc* downstream genes are several adhesion molecules such as integrins [72] and CD44 [130] and a general role for c-*myc* in the control of cell adhesion can be inferred from several indirect or correlative findings. For example, changes in c-*myc* expression have been seen to occur in parallel with changes in fibroblast cell adhesion [131], and in B-lymphoblastoid cells, c-*myc* can influence homotypic adhesion through regulation of the integrin LFA-1 [72]. In estrogen receptor positive breast cancer cells, estrogen stimulates both c-*myc* expression and adhesion to substratum.

There are at least three different mechanisms by which human breast cancer can achieve increased c-*myc* expression: transcription activation by estrogen, gene amplification, and increased mRNA stability [125]. Since 1986 almost thirty studies have reported c-*myc* amplification in over 5000 tumors, with the incidence ranging from 1-33% with an even distribution that is unrelated to study size. This appears to be a reflection of different genetic composition among study groups but it may also be related to technical variation, such as selection of cases, tissue source and tumor cell content, the use of different control genes and the definition of amplification. Based on microdissection and the polymerase chain reaction based assay it has been shown that c-*myc* amplification can also occur at an early stage of tumor progression, in the *in situ* component of invasive carcinoma [25]. The clinical significance of c-*myc* amplification has been hard to determine because of the relatively small number of cases enrolled in most of these studies. The indication is that the presence of amplification correlates with a number of poor prognostic indicators including high levels of Cathepsin D, but possibly the most consistent and convincing association is with high pathological tumor grade [132]. Several large and mostly retrospective studies have also concluded that alteration of c-*myc* is associated with relapse-free survival and overall survival. In human breast cancer, c-*myc* amplification and increased expression are associated with high grade tumors and the most aggressive form of breast cancer, inflammatory carcinoma.

As described earlier, it is quite possible that c-*myc* expression might become altered by a number of mechanisms apart from amplification [133]. These might include changes in chromatin structure in the promoter region [134] or gene structure through translocation. This has been described in some cases but appears to be uncommon [135-

136]. However, the existence of altered regulation at other levels such as changes in the regulation of transcription, mRNA stability or titration of the activity of the protein through levels of *Max* protein and other partners has not been well investigated. This is partly due to the fact that many expression studies are difficult to interpret given the marked heterogeneity of c-*myc* expression that has often been documented [133, 137], (both in tumor cells and in relation to infiltrating lymphocytes). Nevertheless it appears that tamoxifen therapy [138] and proliferative status [139] can influence the levels of c-*myc* mRNA and protein. The involvement of c-*myc* in apoptosis makes it possible that expression of this gene will also be one of many factors that affect response to endocrine- or chemo-therapies that rely on the induction of apoptosis [140].

The *in vivo* associations between alteration of c-*myc* with high grade tumors, early recurrence and poor prognosis [141] but less consistently with nodal status or metastasis [25] is in keeping with a specific role in invasion. According to one study, c-*myc* amplification is a better prognostic marker than is c-*erb-2* [142].Thus both *in vitro* and *in vivo* studies indicate that alteration of c-*myc* may be an important factor not only in initiation but also in some aspects of breast tumor progression and that this gene may for example play a role in the transition from hormone dependent to hormone independent growth.

1.4 The Estrogen Receptor Gene:

Steroids are potent signal transducers that act to coordinate biological functions related to cell development and differentiation; their biological effects are potentiated through protein receptors. Extensive research has been done on the structure/function of these receptors and their mechanism of gene regulation [143]. All steroid receptor

complexes contain untransformed aporeceptor and a 90kDa heat shock protein (*hsp90*) [143]. A 59 kDa protein is also loosely associated with the complex [144]. In addition to *hsp90*, certain receptor complexes may also contain another heat shock protein, *hsp70*, and uncharacterized 56, 54, 50 and 23 kDa proteins [145]. In all cases receptors of these oligomeric complexes do not interact with DNA.

The estrogen receptor (ER) gene has been cloned from human, chicken, mouse, rat and *Xenopus laevis*. In all cases there is a single mRNA of between 6.2-7.5 kb that codes for a 589-600 amino acid protein with a molecular weight of about 66.5 kDa. All ER mRNA contain a large 3' untranslated region the significance of which is unknown. All receptors cloned are remarkably similar indicating a strong evolutionary bias towards maintaining the ER unchanged and underlies its importance in the basic development of various cells. Once transcribed, capped and polyadenylated, the ER mRNA, like most eukaryotic mRNA is transported into the cytoplasm to undergo translation. This nuclear translocation may occur non-specifically by diffusion of the receptors through the nuclear membrane or specifically by nuclear pore protein-receptor interactions [146]. This later mechanism requires that the translocated protein contain a specific nuclear translocation signal sequence. Such a sequence may be present in the ER from amino acids 256-303 [147].

Structural and functional characteristics :

Based on sequencing data, the receptors could be subdivided into regions A-F [148-150], Figure 1.3. The genomic organization of the ER has revealed that the A/B region and the 5' untranslated sequences are encoded by the first exon, the DNA binding C region is encoded by the exons 2 and 3, each exon encoding one of the zinc fingers,

while the remaining 3 structural domains and the large 3' untranslated region are encoded by the remaining 5 exons [149]. The various functions that are associated with the functional domains of ER are responsible for transcriptional activation and repression, nuclear localization, DNA binding and hormone binding of the receptor. The least well conserved region is the N-terminal A/B region, which contains a constitutive activation function that activates transcription of estrogen responsive genes in a cell type specific manner [151]. The next region amino acid domain C, is highly conserved, and contains 20 invariant residues including 9 cysteines, which fold in 2 zinc-finger DNA binding motifs. This DNA binding domain (DBD) is responsible for the recognition of the specific enhancer sequences found in hormone responsive genes, generally referred to as hormone response elements (HREs). Each receptor recognizes its own response element, so that extra-cellular hormonal signal are transduced via the receptor to specific target genes. Discrimination between different HREs is determined by three amino acids at the base of the first zinc-finger [152], and single base pair substitution can produce receptor that recognize different HREs. Thus, sequence mutations within this region could have important functional consequences. The C-terminal extremity of the DBD of the ER also contains a nuclear localization sequence (NLS) important for the nuclear localization of the receptor [153].

The DNA binding domain C is separated from the large hydrophobic E domain by a hinge of variable length and amino acid composition (the D region). Several *in vitro* substitutions within this single domain have had little effect on ER function [149]; however, this result must be interpreted with caution because one report suggests that a single mutation within this region inactivates the related v-erbA nuclear receptor [154].

The E domain is a complex region containing the hormone binding site and a region required for stable dimerization of the receptor [155]. In addition, this region contains a transcriptional activation function which is hormone inducible, and which synergizes with the A/B region transactivating activity depending on target gene promoter context [151]. These studies indicate that the E region is very complex, and mutations within this region of the receptor could have profound consequences on receptor function.

Molecular mechanism of estrogen action:

Estrogen is known to be etiologically important in the development of breast cancer. Estrogen's effect on target organs is mediated through its receptor, and in the case of breast epithelium, it appears that estrogen effect includes the induction of proliferation, particularly of ductal tissue [156]. In breast cancer cells several classes of genes are candidates for both direct and indirect roles in the growth response to estrogen. These include nuclear oncogenes such as c-*myc*, c-*fos* and c-*jun* which are estrogen regulated in other systems, 'house-keeping' genes involved in nucleic acid metabolism, the ER and several secreted proteins such as cathepsin D and growth factors [8-11]. Estrogen also induces the expression of other genes which have no clear role in the growth response, such as the progesterone receptor (PR) and the secreted protein pS2. The presence and concentration of estrogen receptors in the breast tumor is widely used as a clinical index of potential therapeutic response to endocrine therapy. One half of ER positive tumors respond well to endocrine therapy, however in most instances this initial success is eventually followed by the development of resistance to this treatment. To understand the mechanism of this hormone-dependent to hormone-independent transition, detailed molecular analysis of pathologically defined human tumor material is important.

1.5 Research Objectives and Significance of this Study:

In vitro studies have demonstrated that c-*myc* is a direct target of estrogen action and structural alteration of c-*myc* may exist in breast tumor tissues and cell lines. However, at present no data are available on the effect of time of specimen collection on the decay rate of the unstable c-*myc* m-RNA. The main aim of the present study is to obtain this information and to fill this gap in the literature. The long term goal of our laboratory is to determine if alteration of c-*myc* expression *in vivo* is a factor in tumor progression and the development of resistance to hormone therapy.

The detection of specific RNA transcripts is widely used to monitor gene expression. It is well known that the half-life of different mRNA species may vary from minutes to several hours. While these differences will leave little influence on the assessment of gene expression in cell lines studied under controlled conditions, the impact of this variable on gene expression studies performed on human tissues that often differ in their timing and method of collection has rarely been considered.

The conventional methods of RNA analysis such as northern blot hybridization followed by densitometric quantitation require 10-40 μg total RNA. However, studies with human samples are often limited by availability of sufficient quantities of tissue. Techniques such as *in situ* hybridization or RNase protection assay are more sensitive and thus may be used with small samples, but it is difficult to obtain quantitative results using these methods. Therefore the main objectives of this study were:

1. To develop a sensitive and reliable method for absolute quantitation of c-*myc* and estrogen receptor gene expression (quantitative RT-PCR) that could be applied to a small amount of surgically resected human breast cancer specimens.

2. To validate the sensitivity of RT-PCR assay against conventional methods such as Northern analysis.
3. To determine the effect of timing of tissue collection on the decay rate of unstable c-*myc* mRNA in human breast cancer, and to compare the results with those on the effect of timing of tissue collection on the level of more stable ER mRNA.
4. To use the results as a basis for interpretation of future studies on the correlation between changes in c-*myc* gene expression level, hormone response and tumor progression.

Chapter 2

Materials and Methods

2.1 Cell Line RNA:

Breast cancer cell lines including MCF7, T47D, MDA MB-231 (ATCC) were used to standardize the RT-PCR assay. All cells were grown under standard conditions (DMEM+5% fetal calf serum). MCF7 cells were then deprived of steroids by growth in charcoal stripped serum followed by subsequent treatment with estradiol (10^{-9} M) or tamoxifen (10^{-7} M) to induce or repress c-*myc* expression as previously described [12]. The MCF7 cell line RNAs were provided by Dr. Don Dubik (Dept. of Physiology, University of Manitoba).

2.2 Human Breast Cancer Samples:

The surgically resected breast carcinoma samples were obtained in conjunction with the NCIC-Manitoba Breast Tumor Bank at the Health Sciences Center (HSC) Hospital, Winnipeg, Manitoba. The specimens were collected and rapidly brought on ice from the operation room to the department of pathology, minced thoroughly with a scalpel blade on ice (to take into account the issue of tumor heterogeneity), aliquoted and stored for variable periods of time at 4 °C on ice until put in -70° C freezer at specific times after surgery. This protocol closely follows the handling of specimens for the Tumor Bank. The project time 0 hour specifies the time at which the first tissue aliquot from each tumor was put at -70° C, and the subsequent 'project times' were measured from this time. The actual times after surgery are indicated as 'real time' in Figure 2.1. A

tissue sample adjacent to the original specimen was fixed and processed for formalin fixation and paraffin sections by the Manitoba breast tumor bank technologist. This allowed high quality histological sections to be assessed and pathological interpretation of the immediately adjacent tissue used for this study. Approximately 75 tumors are collected at HSC per year and the tissue from each is used for clinical, histopathological and other analyses such as determination of ER and PR values. Therefore the tumor samples could be collected only from the specimens which were big enough to provide enough tissue for this research project and 11 samples were collected in a time period of about one year.

2.3 RNA Preparation:

Total RNA from tumor samples was extracted using TRI reagent method (Molecular Research Center, Inc., Cincinnati). The TRI reagent (1 ml), containing phenol and guanidine thiocynate in a mono-phase solution, was added to the tumor tissue samples (50-100 mg) and homogenized using a polytron homogenizer. The homogenized samples were stored for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added per 1 ml of TRI reagent. The samples were covered and mixed vigorously for 15 seconds and stored at room temperature for 2-3 minutes. The resulting mixture was centrifuged at 12000 g for 15 minutes at 4°C. The upper aqueous phase containing RNA was transferred into fresh autoclaved tube. 0.5 ml of isopropanol per 1 ml TRI reagent (used for initial homogenization) was added, mixed and stored at 4°C for 30 minutes. The mixture was centrifuged at 12000 g for 10 minutes at 4°C. The supernatent was removed and the RNA pellet was washed once with 1 ml of 75% ethanol by vortexing and subsequent centrifugation at 7500 g for 5 minutes at 4°C. The RNA pellet was briefly dried by

air drying on ice and then dissolved in RNase free diethyl-procarbonate (DEPC) treated water. The yield was quantitated by spectrophotometry at 260 nm.

2.4 Primer Design for c-*myc* Fragment:

The following oligonucleotides (Gene Bank accession number J-00120) were used as primers for cDNA synthesis and PCR amplification of specific c-*myc* fragments from MCF7 human breast cancer cell line RNA.

Primer name	oligonucleotide sequence	position
MYC P1	5' ACC ACC AGC AGC GAC TCT 3'	2639
MYC P2 & P4	5' GTT CGC CTC TTG ACA TTC TC 3'	2952
MYC P3	5' TGA CAT TCT CCT GAC ACT GTC CAA CTT GAC C 3'	2870

The other sequences appended to *MYC* P1 and *MYC* P4 primers (Figure 2.2) were:

T7 Promoter sequence	5' TAA TAC GAC TCA CTA TAG G 3'
Eco RI	5' GAA TTC 3'
T18	5' TTT TTT TTT TTT TTT TTT 3'
Bam HI	5' GGA TCC 3'

The three step RT-PCR strategy used in this component of the present study (Figure 2.2) was similar to that previously described [157]. In this method a homologous competitive fragment is created that is smaller than the wild type gene sequence.

The initial RT-PCR was carried out with human breast cancer cell line RNA. After the first PCR with *MYC* P1 and *MYC* P2 primers, a 15 μl aliquot of the reaction mixture was analyzed by gel electrophoresis and the band of expected size (333 bp) was detected. In the first reamplification step 1μl of a 1:1000 dilution of the mixture of previously amplified PCR product was used as a template and the 3' primer (P2) was replaced by an internal linker having a tail of 10 bases at the 5' end that are identical to the first 10 bases at the 3' end of the *MYC* P2 primer and thus serve as a linker between

those two primers in the second reamplification step. In both the reamplification steps, the same 5' primer as for the 333 bp fragment was used. After the first reamplification a 15 μl aliquot of the reaction mixture was analyzed by gel electrophoresis and only the band of the expected size (286 bp) was detected. This also served as control for the correct base sequence of the PCR fragment to be quantified. In the second reamplification 1μl of a 1:1000 dilution of the reaction mixture containing the 286 bp fragment was reamplified with the original 3' primer. By analysis through gel electrophoresis only one band slightly bigger than the template PCR product was detected. This 310 bp PCR product was then digested with EcoRI and BamHI and subcloned into pSG5 vector (Stratagene, La Jolla, Ca) between the T7 promoter and poly (A) tail.

2.5 Primer Design for Estrogen Receptor Fragment:

Another strategy (Figure 2.3), was used for the construction of the fragment for ER synthetic standard [158]. In this method amplification of a heterologous 'spacer' gene sequence (c-*myc* intron1 sequence) with primers containing 5' appended sequences for the T7 promoter, EcoRI, target gene mRNA PCR primer sequences, BamH1 sequence and poly (dT) was performed. The PCR product was analyzed by agarose gel electrophoresis and then digested with EcoRI and BamH1 and subcloned into pSG5 vector between T7 promoter and poly (A) tail. The oligonucleotides (Gene Bank accession number M-12674) used as primers were:

Primer name	oligonucleotide sequence	position
MYC A	5' CGC GAC TCT C 3'	2746
MYC B	5' CGT CCT TAC C 3'	2973
ER 5	5'TGC TCC TAA CTT GCT CTT GG 3'	8
ER 7	5' TCC AGA GAC TTC AGG GTG C 3'	65

2.6 The Scheme for Construction of c-*myc* and ER Synthetic Standard cRNA:

The final PCR product of c-*myc* and ER synthetic standards were digested with EcoRI and BamHI, purified from a on 6% polyacrylamide gel and subcloned into an EcoRI/BamHI digested pSG 5 vector (Stratagene) shown in Figure 2.4. The c-*myc* cRNA and ER cRNA were synthesized from xba1 linearized pSG5-*cmyc* plasmid by T7 polymerase using Promega *in vitro* transcription kit. The following reagents were added at room temperature: 20 μl of transcription optimized 5x buffer, 10 μl of 100 mM DTT, 100 unit (2.5 μl) of RNasin Ribonuclease inhibitor, 2.5 mM of each rNTP (ATP, GTP, CTP, UTP), 10 μl of linearized DNA template (1-2 mg/ml in double distilled and autoclaved water), 15-20 units/μl of T7 polymerase and nuclease free water added to make a final volume of 100 μl. The reaction mixture was incubated for 120 minutes at 22°C.

The template DNA was degraded with RQ1 RNase free DNAse (Promega) and the synthetic standard was extracted by buffer-saturated phenol/chloroform (24:1). The upper RNA containing phase was transferred to a fresh tube and extracted with chloroform/isoamyl alcohol (24:1) followed by precipitation of aqueous phase with ethanol. The cRNA was washed with 70% ethanol and suspended in nuclease free water and quantitated by absorbance at 260 nm.

2.7 Competitive RT-PCR:

cDNA preparation: RT-PCR conditions such as magnesium chloride ($MgCl_2$) concentration, annealing temperature (AT) and cycle number were first optimized for

each primer set. Aliquots of total endogenous RNA (50 ng) were prepared and a dilution series of the cRNA standard (2 fold dilution from $4x10^{-2}$ to $6x10^{-4}$ ng) was spiked into these aliquots. Reverse transcription of RNA was performed in a final volume of 20 µl containing 0.05 M Tris HCl (pH 8.3), 0.075 M KCl, 3 mM $MgCl_2$, 100 mM dNTPs (A, G, C and T), 1 mg/ml BSA, 0.01 M DTT, 2.5 mM oligo dT (for c-*myc*) or 2.5 mM random hexamer primer (for ER), 200 U MMLV-RT (GIBCO-BRL). The reaction mixture was incubated at 37°C for one and a half hours and then diluted with double distilled and autoclaved water to a final volume of 40 ml.

PCR amplification: One twentieth (2 µl) of the serial RT reactions containing both endogenous+standard cDNA's was then added to 48 µl of master mix, amplified for c-*myc* or ER in a prechilled 50 µl reaction mixture containing 0.5 mM short *MYC* P1 (with no T7 and EcoR1 Sequence) and *MYC* P2 primer, 0.10 mM dNTPs (A, G, C, T) 1 mM Tris-HCl, pH 9 at 25°C, 5 mM KCl, 1% Triton x-100, 1 ml DMSO F.W 78.13 and 1.1 units of Taq Polymerase (Promega). The mixture was overlaid with 50 ml of mineral oil and amplified in a thermal-cycler (MJ Research) with a hot start for a total of 30 cycles with the following temperature settings. Initial denaturation 94°C for 5 minutes, followed by 30 cycles of 93°C for 45 seconds, 56°C for 45 seconds, 75°C for 1.3 minutes, after completion of cycles 75° C for 7 minutes and reactions held at 72° C until being placed on ice for gel analysis. For ER experiments 0.5 mM ER 5/7 primers were used with $MgCl_2$ concentration of 1.5 mM and cycle number 35.

2.8 Quantitative Image Analysis:

Using the internal control RNAs described above we quantitated c-*myc* and ER mRNA level in human breast cancer cell lines and tumor tissues. The strategy involved

simultaneous reverse transcription of internal control RNA and unknown amounts of target mRNA followed by simultaneous PCR on specified amounts of cDNA.

One-third (15 ml) of each PCR product was size fractionated through a 3% agarose gel. (Boehringer Mannheim). The 72 bp difference in length for c-*myc* (wild type target c-*myc* RNA of 333 bp and synthetic standard of 261 bp) and the 78 bp difference in length for ER (wild type 198 bp and cRNA 276 bp) allowed these bands to be easily distinguished on the ethidium bromide stained agarose gel visualized under UV light. The intensity of the two bands corresponding to cellular (endogenous) and competitor (synthetic standard) amplification products was directly measured from the UV illuminated gel on image analysis system, consisting of a charge-coupled device (CCD) video camera connected to a Dell Pentium 100 Mhz computer. Image processing and analysis were performed with the MCID-M4 software (Imaging Research Inc.). Fluorescence was recorded and intensities of the bands corresponding to the target and competitor were measured, plotted on a graph and the point of equivalence determined for each sample, i.e., the point at which the level of target mRNA was same as that of the internal standard. This provided the basis of quantification.

2.9 Northern Analysis:

It was possible to check the integrity of tumor RNA by Northern analysis in the 3 largest tumor specimens that yielded sufficient RNA for this type of analysis. At each time point 10 μg of each sample from time 0, 3, 6, 24 hours project time from three tumor specimens was fractionated under denaturating conditions on a 1% agarose, 2.2 M formaldehyde denaturing gel and analyzed under UV light. The Northern blot with MCF7 RNA corresponding to RNA samples used for standardization of the c-*myc* assay was provided by Dr. Don Dubik (Department of Physiology, University of Manitoba).

Chapter 3

Results and Discussion

3.1 Internal Standard:

The PCR is an exponential amplification and even small variations in amplification efficiency result in dramatic changes in product yields. The use of internal controls that contain the same primer template sequences as the target makes it possible to determine the absolute amount of target cDNA by allowing known amounts of competitor to compete with the target for primer binding during the amplification [159]. In the present study, we have included an internal control RNA to overcome the problem of efficiency in quantitating c-*myc* and ER mRNA levels at the RT as well as the PCR step. The internal control for the c-*myc* assay was made by overlap extension PCR, and for the ER assay by a different method using a spacer gene sequence with primers containing 5'-appended sequences for the T7 promoter, target gene mRNA PCR primer sequences and poly (dT) the principles of which are shown in Figures 2.2 and 2.3, respectively. The RNA was transcribed from the synthetic sequence cloned into the vector pSG5 using T7 polymerase, according to manufacturer's (Promega) instructions.

The ideal competitor should have following properties: it should be readily prepared, easily distinguishable from the target, amplify with equal efficiency, provide a result that is independent of cycle number and does not require radioisotopes for detection. The commonly used competitor types are deletion type, mutant type, intron type and nonhomologous competitor. Each of these methods have been evaluated for their

potential advantages and disadvantages, such as their ability to amplify the target with the same efficiency, as well as their capacity to form heteroduplexes with it [160]. Even small differences in amplification efficiencies that might arise from using nonhomologous template could in theory translate into significant variations in product yields in a typical PCR of 30-40 cycles. Therefore while measuring rare species of mRNA, which require greater amplification, any small differences in efficiencies need to be minimised. Thus when constructing a competitor it is important to minimize size difference with the target. In a recent review, Clementi and coworkers [161] have also emphasized the need to minimize the differences when constructing deletion or insertion type competitors. When using a mutant type competitor as an internal standard, amplification efficiencies are the same but digestion-resistant heteroduplexes arise as the reaction approaches the plateau phase and may result in the equivalence point becoming cycle dependent. A recent study [162] has also shown formation of heteroduplexes when using a deletion construct in a competitive PCR assay. Therefore a potential disadvantage of using a mutant competitor is that the analysis can be dependent on the degree of heteroduplex formation.

The two methods used for the generation of internal standards for c-*myc* and ER assays have their own advantages and both are rapid and versatile. The reamplification step, in the three step PCR method to construct a homologous standard has the advantage of serving as control for the correct base sequence of this PCR product and unwanted sequences can not interfere with the internal primer. Furthermore, in this method the size of the internal primer can be much shorter because no interference with unwanted sequences is possible; thus the internal primer will be less expensive. The PCR products analysed by gel electrophoresis can be excised immediately for use as a template. The use

of spacer gene primer sequences in the second approach used for generation of ER internal standard allows one to construct an internal standard that has a convenient PCR product length and can be easily adapted to any target gene sequence. Since the spacer gene may be any sequence that can be amplified by PCR, one can easily design internal standards for RT-PCR fragment of any size. Thus this method has an additional advantage of flexibility for inclusion of sequences within the internal standard. This sequence can be an internal hybridization sequence which can allow southern blot hybridization detection of PCR products from both cRNA and target gene sequences.

The two strategies using homologous and heterologous competitive standards for c-*myc* and ER assays worked equally well in the present study. However, the initial idea of appending T7 promoter sequences at the 5' end of forward primer and poly dT at the 5' end of the backward primer for in vitro transcription of the c-*myc* and ER fragments was not successful (possibly due to poor efficiency of the limited T7 promoter sequence in the primer). To overcome this the fragments were finally subcloned into pSG5 vector having a full T7 promoter sequence and a poly A tail (Figure 2.4). The EcoRI and BamH1 sites were used for correct orientation of the fragments into the vector.

It was found necessary to stringently purify the synthetic standard control from DNA template fragment by RQ1RNnase free DNase treatment since a false positive signal unrelated to reverse transcription efficiency would result from even a trace amount of contaminating DNA template. The absence of DNA template from the internal RNA standard was tested by performing 35 cycles of PCR on RT reactions which lacked RTase enzyme, and no evidence of DNA contamination was seen. After a typical reaction to produce synthetic standard c-*myc* cRNA and ER cRNA *in vitro* yields obtained and

assessed by spectrophotometric quantification at 260 nm were 2.8 mg of c-*myc* standard from 10 mg of recombinant pSG5-*myc* plasmid construct and 4 mg of ER cRNA from 10 mg of recombinant pSG5-ER construct.

3.2 Rate of Amplification:

To determine the optimum number of PCR cycles, $MgCl_2$ and annealing temperature required to keep the amplification in the exponential phase and also to amplify only the specific product, a known amount of specific internal control RNA was reverse transcribed and amplified using c-*myc*/ER primers. The PCR products obtained were analyzed on the ethidium bromide stained 3% agarose gel. The experiment was repeated using different amounts of endogenous RNA with serial dilutions of the synthetic internal control during RT-PCR to see the optimum concentration of endogenous RNA that could influence the exponential nature of the competitive RT-PCR. This experiment showed that the optimum number of cycles fell between 30-45 cycles. Since it is advisable to keep the PCR cycles to the minimum number required in order to avoid nonspecific amplification, 30 and 35 cycles were chosen for c-*myc* and ER quantitation experiments. The optimum $MgCl_2$ concentration for c-*myc* RT-PCR was 2.5 mM and for ER 1.5 mM, optimum AT for both was 56 °C.

3.3 Sensitivity and Range of the Assays:

After checking the purity of internal standards for c-*myc* and ER, RT-PCR was performed to test if the endogenous c-*myc* and ER PCR products could compete with the respective cRNA standard signals in human breast cancer cell lines and tumor RNAs. As shown in Figures 3.1 and 3.2 the bands for endogenous PCR products could be well

distinguished on a 3% agarose gel from the bands of the internal standard. In addition, the band intensities from the products of the competitive reactions reveal that products from the cRNA internal standard decreases as the concentration of the spiked cRNA internal decreases. The PCR product from the endogenous RNA increases as the PCR product from the internal standard decreases with the equivalency point occuring where the level of the endogenous RNA is the same as the amount of internal standard added in that tube.

Sensitivity of the c-*myc* assay was first tested against Northern blot analysis performed with a panel of MCF7 breast cancer cell line RNAs (Figure 3.3). The result of RT-PCR assay corresponded very well to that of Northern analysis done with the same RNA samples. The c-*myc* expression being highest in the estrogen treated sample and lowest in tamoxifen treated sample (Figures 3.4 a and b). The c-*myc* expression in estrogen treated MCF7 sample was 7.9×10^{-3} ng, in cells grown in normal fetal calf serum (FCS) was 3.8×10^{-3} ng, in cells grown in charcoal stripped FCS medium was 1.8×10^{-3} ng and in tamoxifen treated samples was 0.9×10^{-3} ng. The corresponding levels as measured by computerized image analysis of the Northern blot done with the same RNA samples (Figure 3.3) were 0.5354, 0.3846, 0.3121, 0.2792 scan density units respectively. A calibration graph was plotted between RT-PCR assay and Northern analysis taking all the results relative to that of T180 sample (Figure 3.5) for standardization of further results with the human breast cancer specimens. The c-*myc* assay could accurately distinguish a minimal 2 fold difference by comparison with Northern blot analysis. The percentage reduction in *myc* and ER levels was calculated from the slope of the calibration graph using the formula (1-value at 0 hour/value at n hour x 0.0566) x 100.

The sensitivity of the ER assay was then assessed using estrogen receptor positive

RNA that had been extracted from an ER+ve tumor with ER value of 211fm/mg of protein and mixed with ER-ve (MDA231 cell line) RNA in the following ratios: 100:0, 75:25, 50:50, 25:75, 10:90 and 1:99. The ER+ve tumor with ER level of 211fm/mg was used because it relates to the range normally found in high ER+ve tumors (usually 25% ER-ve, ER value <3 fm/mg protein and 75% ER+ve, ER>3 fm/mg protein, high level could be regarded as >100). The competitive RT-PCR was done as before using endogenous RNA sample (50 ng) with decreasing amounts of synthetic standard. Figures 3.6 a and b show that the point of equivalence shifted towards the right across the panel of samples, indicating a decrease in the ER expression from ratio 100:0 to 1:99. These results (Figure 3.7) illustrate that the ER RT-PCR assay can distinguish at least a 100 fold difference in ER levels in a mixtures of ER+ve and ER-ve RNA samples.

3.4 Reproducibility of the Assay:

We observed very little variation in the assay, when performed on the same sample on different occasions eg. the c-*myc* mRNA levels observed in 0, 3 and 6 hour samples of tumor number 7 gave the values of 10^{-4}, 10^{-4} and 2×10^{-4} ng on one occassion and 1.4×10^{-4}, 1.3×10^{-4} and 2×10^{-4} ng on second occassion. To minimize variability due to experimental conditions, the all time points for each tumor specimen were done in the same assay on the same day under similar conditions.

3.5 Integrity of Tumor RNA:

To check the integrity of RNA in tumor samples (cases 2, 8 and 9), 10 µg of each time point sample was run on a 1% Northern gel. Figure 3.8 indicates that there was no significant degradation of tumor RNA at each time point as assessed by subjective

visualization of the presence and intensity of the 28 S and 18 S ribosomal RNA bands. This confirmed that the condition of the tumor RNA was probably equivalent in quality and good for quantitation of c-*myc* and ER mRNA levels. Therefore the % reduction of c-*myc* mRNA levels over 24 hour time period which was considerably higher in case numbers 2 and 9 as compared to that of tumor number 8 (Table 3.2, Figure 3.11) is unlikely to be attributable to differences in RNA quality.

3.6 Effect of Tumor Collection Time on c-*myc* and ER mRNA Levels:

The competitive RT-PCR assay was performed with RNA extracted from tumor samples frozen at project times 0, 3, 6 and 24 hours. The gel pictures for *myc* and ER assay for one representative tumor specimen are shown in Figures 3.9 a and b. The results (Figures 3.10 & 3.11 and Tables 3.1& 3.2) obtained by analyzing 10 human breast cancer specimens showed a decline in the rate of c-*myc* mRNA over 24 hours with a standard deviation (sd) of 22.6% and a mean value of 74.7% (of the level at time 0 hour). The decline in ER mRNA levels showed smaller change 91% to 98% of the level at 0 hour (Figures 3.12 & 3.13 and Tables 3.3 &3.4) with a sd of 3.2 and a mean of 95% (of the level at time 0 hour).

The result of the c-*myc* mRNA quantitation assay done on different time point tumor RNAs were compared with that of ER. The results of assessment of *myc* and ER mRNA levels done on tumor samples from cases 2, 4 and 6 show that the decline in c-*myc* mRNA over 24 hours was large with the reduction ratios (level at time 0 / level at 24 hour) of 7.25, 7.87 and 1.16, respectively, as compared to 0.35, 1.52 and 1.0, respectively

for ER. The sample number 3 shows a comparatively large reduction in c-*myc* level (Figure 3.11). The subsequent histopathological examination revealed that this sample, obtained from the margins of a previous biopsy site, contained inflammatory, fibrotic and necrotic tissue but no residual tumor. The presence of necrotic tissue might have been responsible for the rapid degradation of RNA over 24 hours.

3.7 Histopathological Assessment of Tumors:

The differences observed in the reduction of c-*myc* mRNA levels in different tumors assessed in the present study showed no direct relationship with the clinical and histopathological features (Table 3.5) as assessed in adjacent paraffin blocks. Statistical analysis was performed to determine if there was any relation between initial c-*myc* level at time 0 hour with the ER and PR status of the tumors. Student's t-Test were conducted on c-*myc* levels at time 0 hour (Tables 3.1 and 3.3) with ER+ve/-ve as well as PR+ve/-ve values of the tumors (Table 3.5), to determine if the mean values of the data sets are significantly different (by testing the hypothesis that the means of the two columns are equal). No significant difference was observed in the starting c-*myc* levels in ER +ve/-ve tumors. The 3 PR+ve tumors showed low levels of c-*myc* at time 0 hour, whereas, 6 out of 7 PR-ve tumors showed relatively higher levels of c-*myc* (2 to 5 fold higher), $t = -4.98$, $P = 0.00032$, (where t is Student's t statistics, P is the probability that one is incorrect in stating that the two means are different). The following two qualitative observations may be made from the above results: (1) The ER mRNA levels assessed in 4 tumors correspond to the relative ER status determined by the dextran coated charcoal (DCC) ligand binding assay. (2) The single case of a lobular carcinoma showed higher starting level of c-*myc* than the ductal carcinomas.

Chapter 4

Summary, Conclusions and Suggestions for Future Work:

The knowledge of specific genetic changes and their biological consequences is critical to an understanding of the natural history of breast tumors and the development of rational means to prevent and treat them. The clinical management of breast cancer patients is guided by measures of tumor growth and metastatic potential. The biological factors that affect overall tumor growth, response to signals encouraging cell proliferation, programmed cell death, and cell differentiation, that occur over time lead to unrestricted growth, invasion, and metastasis. The emergence of estrogen independent growth represents one aspect of tumor progression and may be attributable to ascent of an existing hormone dependent phenotype within a heterogeneous tumor, and/or adaptation of the hormone dependent phenotype leading to short circuit in the requirement for estrogen. A better understanding of this process of hormonal progression is dependent on the study of the components of the cellular mechanisms of estrogen action. In laboratory studies of human breast cancer cell lines it has been shown that the c-*myc* gene is critical for the growth of breast cancer cells [123] and that it is directly stimulated by estrogen in ER positive hormone dependent breast cancer cells [12, 122]. In contrast, the expression of c-*myc* is high and constitutive in ER negative hormone independent cells [124]. Several expression studies have also demonstrated that both c-*myc* amplification and altered mRNA stability may exist in breast tumor tissues and cell lines [12, 25, 125]. Thus both *in vitro* and *in vivo* studies indicate that c-*myc* is a direct target of estrogen

action and that alteration of c-*myc* expression may be a factor in breast tumor progression from hormone dependent to hormone independent growth.

Messenger RNA (mRNA) degradation in eukaryotic cells is a regulated process that can determine the level of expression of a gene. In particular, the existence of highly unstable RNAs allows for rapid and precise reductions or elevations in transcript levels following changes in transcript production rates or alterations in the machinery dedicated to transcript degradation. To date most studies of the regulation of eukaryotic mRNA levels have focused on the control of gene transcription. However, the level of a cellular mRNA actually represents a balance between its rate of synthesis and degradation [163]. The range of mRNA stability in eukaryotic cells vary over several orders of magnitude. For instance, in higher cells some mRNA are degraded with half-life of about 20 minutes, while in the same cell, other mRNA are degraded with a half-lives of over several hours. In some cases, sequence elements that silence destablizing sequences have been found, but in no case has a sequence by itself been found to confer stability to another mRNA. The exceptions to this are regions of RNA secondary structure that can stabilize mRNA fragments produced during the decay reaction and the cap and poly (A) tail of the mRNA. A useful working hypothesis is that most mRNAs are relatively stable in cells until subject to intramolecular cleavage by an endonuclease or removal of the protective poly (A) tract from the 3' end of the mRNA is likely to be the key step in the degradation of most eukaryotic mRNAs [164]. The 5' and 3' ends of most eukarytoic mRNAs are protected from exonucleolytic attack by the presence of a 5' cap structure [165] and a 3' poly (A) tract [166] complexed to a poly (A)-binding protein [167]. Since the identification of mRNA degradative intermediates has been quite difficult, it is likely that

the degradation of most mRNAs is rapid once initial cleavage occurs. The degradation rates of many eukaryotic mRNAs are determined by the interaction of specific nucleotide sequences and mRNA secondary structures with protein (and perhaps RNA) components of cells. Although rates of mRNA degradation ultimately may be found to result from the interplay of multiple structural and sequences motifs, most of the known determinants of mRNA degradation are located in the 3'-untranslated region. The sequences in the 5'-untranslated region have been found to play a role in the degradation of c-*myc* in some cells, but not all cells [111-112].

Translation and ribosome loading play important roles in the degradation and stabilization of several mRNAs. Steroid hormones also represent some of the earliest agents shown to control the degradation of specific mRNAs and have been shown to regulate the stability of a substantial number of mRNAs. The estrogen stabilization of apoVLDII mRNA and glucocorticoid stabilization of human GH mRNA result in an increase in the length of their poly (A) tail [168-169]. The relatively unstable *Xenopus* albumin mRNA, which is destabilized by estrogen, contains an unusually short poly (A) tail of 17 residues, whose length does not appear to change in the presence or absence of estrogen [170-171]. Also, the effect of estradiol-estrogen receptor complex may be to induce transcription of a gene(s) coding for a protein(s) required for stabilization [164]. The normally rapid degradation of several unstable growth factor mRNAs, such as c-*myc*, also requires protein synthesis. The c-*myc* mRNA degradative system appears to include a nucleic acid component [172].

There have been several studies of c-*myc* gene expression. However, to the best of author's knowledge, there has been no study to consider the effect of collection time on

the rate of degradation of c-*myc* in human breast cancer specimens. To address this question an RT-PCR based assay was developed. The sensitivity and specificity of this assay was tested for the detection of c-*myc* and ER mRNA levels in human breast cancer cell line RNAs, before applying the assay on human breast cancer specimens. The use of PCR to amplify and quantify mRNA contents allows analysis of low levels of gene expression from a small number of cells or tissue samples. Comparative RT-PCR allows satisfactory quantitation and requires that the RT-PCR product from a gene with variable expression be compared with the RT-PCR product from a reference-gene transcript or an artificial mRNA internal standard. A common approach has been to amplify β-actin, β_2-microglobulin, hypoxanthine-guanosine phosphoribosyl transferase or the enzymes dihydrofolate reductase, glyceraldehyde phosphate dehydrogenase have also been used for this purpose. The mRNA levels of these genes may not always remain constant between cell types and are often present at high levels compared to the gene under scrutiny. Furthermore, sample to sample variation of reference gene expression and gene to gene differences in amplification efficiency can introduce considerable error into quantitation schemes utilizing 'housekeeping' genes either in the same reaction tube or in a parallel series of reactions. In competitive PCR, a DNA fragment containing the same primer template sequences as the target competes for primer bindings and amplification. The PCR products are distinguished by size, hybridization, or change in a restriction site. The use of internal controls that contain the same primer template sequences as the target makes it possible to determine the absolute amount of target cDNA by allowing known amounts of competitor DNA's to compete with the target for primer binding during the amplification. However, competitive PCR does not control for difference in efficiency in

the first RT step that can occur between samples. Competitive RT-PCR using internal cRNA standards introduced prior to the RT step is technically more difficult but has the advantage that it controls for the difference in effeciency in the RT step and can give absolute quantitative results.

In the present study, a sensitive and reliable assay was developed for the quantification of c-*myc* and estrogen receptor mRNA expression by quantitative RT-PCR based on the approach originally described by Wang et al. [159]. The synthetic c-*myc* and ER RNAs were designed as internal standards for this assay to control for the efficiency of both the reverse transcription reaction and the PCR. These synthetic RNAs have the same primer sequences as the endogenous/target mRNA, so that there are no differences in primer efficiencies. Some differences in amplification efficiency may arise from the small (50-75 bp) difference in size between synthetic standard and target, however this allows separation of the corresponding amplification products in an agarose gel, easily handled in clinical laboratories. The PCR assay quantitatively measures c-*myc* and ER mRNA with a synthetic c-*myc*/ER complementary RNA (cRNA) added in serial molar dilutions to equal amounts of total RNA extracted from biological specimens. Following amplification, the amounts of products give the initial amount of target mRNA. The products are then resolved on an ethidium bromide agarose gel and the data quantified by computer imaging of the gel. The developed method was evaluated by quantitating human c-*myc* and ER mRNA levels in both cultured cells *in vitro* and then applied to human breast tumor biopsies *in vivo* to determine the effect of collection time on the decay rate of c-*myc* and ER mRNAs.

The results of PCR based measurements of c-*myc* gene expression in a panel of

MCF7 cell lines show good correlation with the studies carried out by conventional Northern blot analysis technique. Further, the PCR-based assay requires less tissue and ensures more reliability in detection of c-*myc* and ER gene expression in a small amount of biopsy specimen because cell and sample composition can be 'factored in'. The precision of the present nonradioactive assay is enhanced by the accurate measurement of the intensity of the bands corresponding to the PCR-amplified products, by using image analysis of fluorescence of ethidium bromide-stained DNA with a video camera and specific computer software. It was possible to obtain a dose-dependent linear response by using a constant amount of cellular RNA and decreasing concentrations of competitor. The proposed method was evaluated for sensitivity and precision and was found to have good reproducibility. The present c-*myc* assay could accurately distinguish a minimum 2-fold difference by comparison with northern blot and the ER assay could distinguish a 100 fold difference in ER level in mixtures of ER+ve and ER-ve RNA.

Our initial assumption was that preservation of tissue is important for reliable analysis of gene expression in clinical samples. However, the level of RNA expression will probably be affected by changes in rate of gene transcription and RNA degradation in surgically resected samples but tissue preservation is probably most important where genes with relatively unstable mRNA and protein are involved. Our results based on assessment of 10 tumor specimens show that the level of the relatively unstable c-*myc* gene mRNA can decay measurably over 24 hours (range 93 to 54% of initial level in tumor specimens) whereas the level of the relatively stable ER mRNA does not change significantly. The latter was true of tumors that showed greater or lesser degrees of c-*myc* decline. These results indicate that the time of collection of human breast tumor samples

can influence the level of measured gene expression for genes which possess relatively unstable mRNA's.

Although the c-*myc* levels were observed to decay consistently in all tumors it could be argued that over the usual time period of collection of samples (up to 1-2 hours) the relative decay in c-*myc* levels are unlikely to significantly affect results of comparison between tumors. However, it should be considered that these rates of decay were measured on tissue samples that were collected on ice immediately following surgery and maintained on ice up to 24 hours. This is the same protocol that is used for tumor specimens collected routinely for the Manitoba Breast Tumor Bank, and therefore it is clear that these types of samples would be appropriate for study of c-*myc* gene expression in human breast tumors. It is possible that specimens collected at room temperature, as is often done elsewhere, may show increased rates of decay over the same time period, However, we have not had the opportunity to test this.

The time point samples used for this study were made homogenous within each case. This is because heterogeneity has been described in breast cancer with respect to cellular composition and also to various characteristics such as amplification of oncogenes, expression of steroid receptors and expression of c-*myc* expression. Thus tumor samples were thoroughly minced on a petridish plate placed over ice to ensure equivalent samples between time points for each tumor. Therefore, it was not possible to accurately measure the tumor cell composition and to compare between tumors where relative percentage content of tumor cells may vary significantly. However, while the rate of decay in c-*myc* and ER levels showed no significant relationship with clinical or pathological parameters, a trend did emerge between the absolute initial level of c-*myc*

and steroid receptor status. No correlation existed between the c-*myc* level and ER status, however, all 3 tumors that were PR +ve showed low initial levels (at time 0 hour) of *myc*, while PR-ve tumors (6 out of 7 cases) showed relatively higher levels of *myc* (up to 2-5 fold higher). Two other observations are also notable. The initial ER mRNA levels determined by the developed RT-PCR assay correspond to the relative ER status of the four tumors where this was assessed. The single case of a lobular carcinoma indicated a significantly higher level of c-*myc* than the ductal carcinomas.

In the future work three aspects of this study can be pursued further. The first is the observation concerning the relationship between c-*myc* level and ER/PR status. We would like to apply the RT-PCR assay developed in the present study to a larger cohort of breast cancer specimens collected by the Tumor Bank, and to assess c-*myc* and ER/PR levels in frozen sections in order to be able to factor in the tumor cell content and other stromal cells of each case. The second is to study c-*myc* decay further in a larger series of cases to investigate why some tumors showed more while others showed less change in c-*myc* mRNA level. Further, it may be important to investigate whether the effect of other factors, such as hormone status, anesthetics and time/duration of surgery, which were not assessed in the present work, can be attributed to the variation in results of various tumors. It would also be interesting to examine whether c-*myc* decline is the same in neoplastic and normal tissues, and in tissues with inflammation and necrosis present. The third aspect of interest would be to compare the decline of *myc* levels in samples stored on ice or at room temperature to ascertain whether room temprature has any effect on this process. The results of these studies may give useful insite into the correlation between c-*myc* and ER gene expression, hormone response and tumor progression.

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Tumor number	0 hour	3 hours	6 hours	24 hours
1	0.00086	0.00083	0.000064	0.00069
2	0.036	0.01	0.083	0.005
3	0.001	0.000071	nd	0.000085
4	0.047	0.02	0.04	0.006
5	0.00075	0.00088	0.009	0.00029
6	0.00075	0.00064	0.00086	0.00055
7	0.0001	0.0001	0.0002	0.00007
8	0.0072	0.0078	0.006	0.0062
9	0.0051	0.0062	0.00071	0.00069
10	0.001	0.001	nd	0.00069

Table 3.1 The level of c-*myc* mRNA in breast cancer specimens determined by quantitative RT-PCR assay and expressed as amounts equivalent to external synthetic standard (pg). nd=not determined.

Tumor number	0 hour	3 hours	6 hours	24 hours
1	100	94	21	93
2	100	79	97	58
3	100	17	nd	31
4	100	86	93	54
5	100	95	99	85
6	100	93	95	92
7	100	94	97	92
8	100	95	93	93
9	100	95	58	57
10	100	94	nd	92
	Mean	84.2	81.6	74.7
	Min	17	21	31
	Max	95	99	93
	Std. dev.	24.2	27.9	22.6

Table 3.2 The level of c-*myc* mRNA in breast cancer specimens expressed as a percentage relative to time '0'. nd=not determined.

Tumor number	0 hour	3 hours	6 hours	24 hours
2	0.00023	nd	0.00025	0.00065
4	0.013	0.045	0.048	0.0085
8	0.0001	0.0001	0.000068	0.0001
11	0.01	0.01	0.04	0.02

Table 3.3 The level of ER mRNA in breast cancer specimens determined by quantitative RT-PCR assay and expressed as amounts equivalent to external synthetic standard (pg). nd=not determined.

Tumor number	0 hour	3 hours	6 hours	24 hours
2	100	nd	95	98
4	100	98	98	91
8	100	94	91	94
11	100	94	99	97
	Mean	95.3	95.8	95
	Min	94	91	91
	Max	98	99	98
	Std. dev.	2.3	3.6	3.2

Table 3.4 The level of ER mRNA in breast cancer specimens expressed as a percentage relative to time '0'. nd=not determined.

Tumor number	myc 0 hour	Reduction ratio	myc % mRNA	ER 0 hour	ER % mRNA	ER value fm/mg	PR value fm/mg	Type	Nodal status	Size cm	Comments
1	0.0009	1.25	93			298	38	D	P	2.8	
2	0.036	7.25	58	0.0002	98	3.3	7.6	D	N	9	B, 7/52
3	0.001	11.76	31			223	2.3	NT			S, 6/52
4	0.047	7.87	54	0.013	91	71	5.2	L	P	4	
5	0.0008	2.6	85			165	23	M	N	2.5	
6	0.0008	1.37	92			1.3	7.7	D	N	6.5	
7	0.0001	1.43	92			22	226	D	N	3.5	
8	0.0072	1.16	93	0.0001	94	11.5	2.7	D	N	5.5	B, 4/52
9	0.0051	1.67	57			2.4	1.9	D	N	5	B, 1/52
10	0.001	7.39	92			1.5	7.2	D	N	5	B, 3/52
11		nd	nd	0.01	97	21	39	L	P	6	

Table 3.5 Correlation of experimental results with the clinical and histopathological data of breast tumor specimens.
B=biopsy, D=ductal, L=lobular, M=mucinous, N=negative, NT=no tumor, P=positive, S=surgery, 7/52=7 weeks.

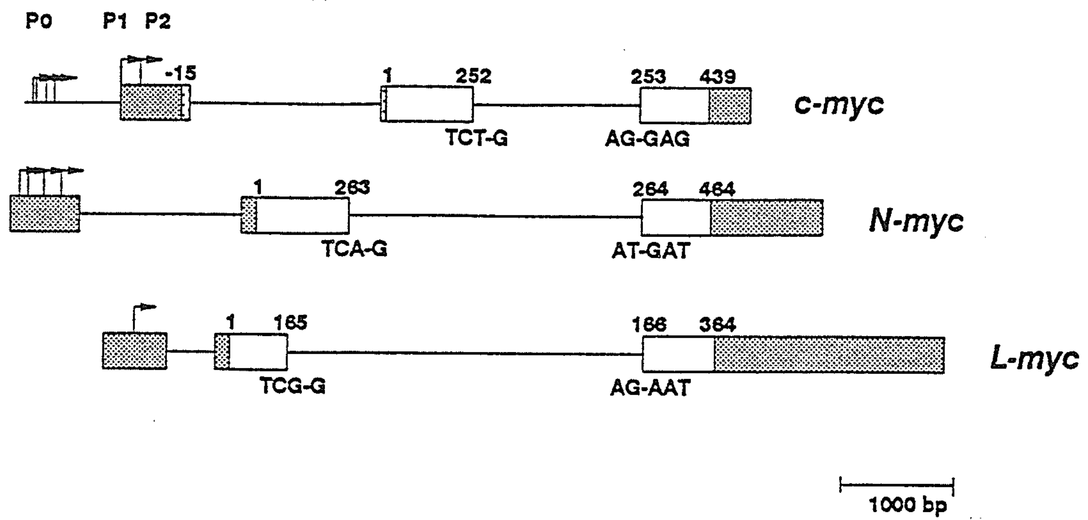


Figure 1.1 Genomic organization of *myc* proto-oncogene family.

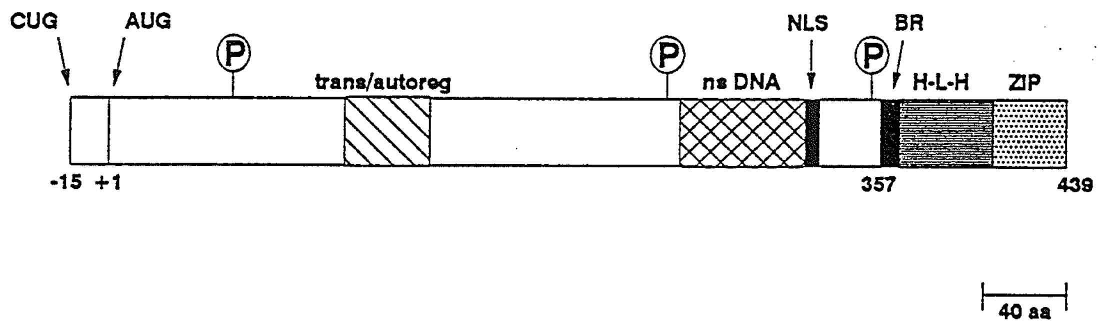


Figure 1.2 Organization of the c-*myc* protein.

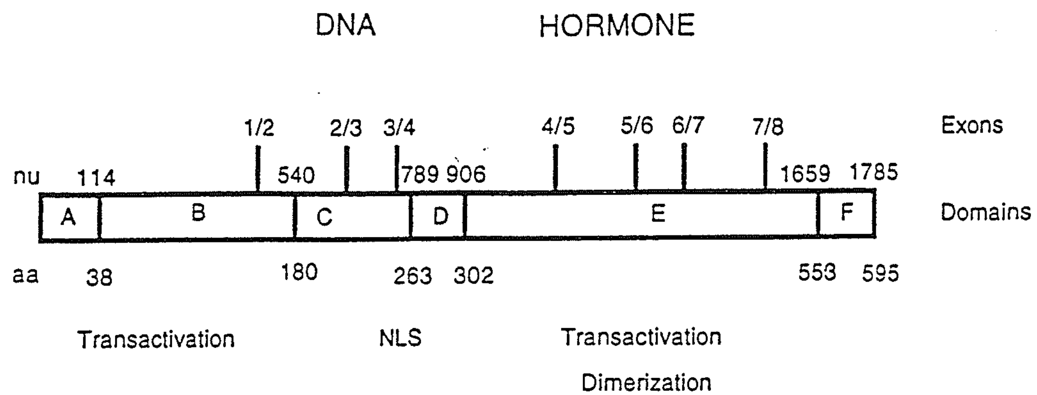


Figure 1.3 Functional domains of estrogen receptor gene.

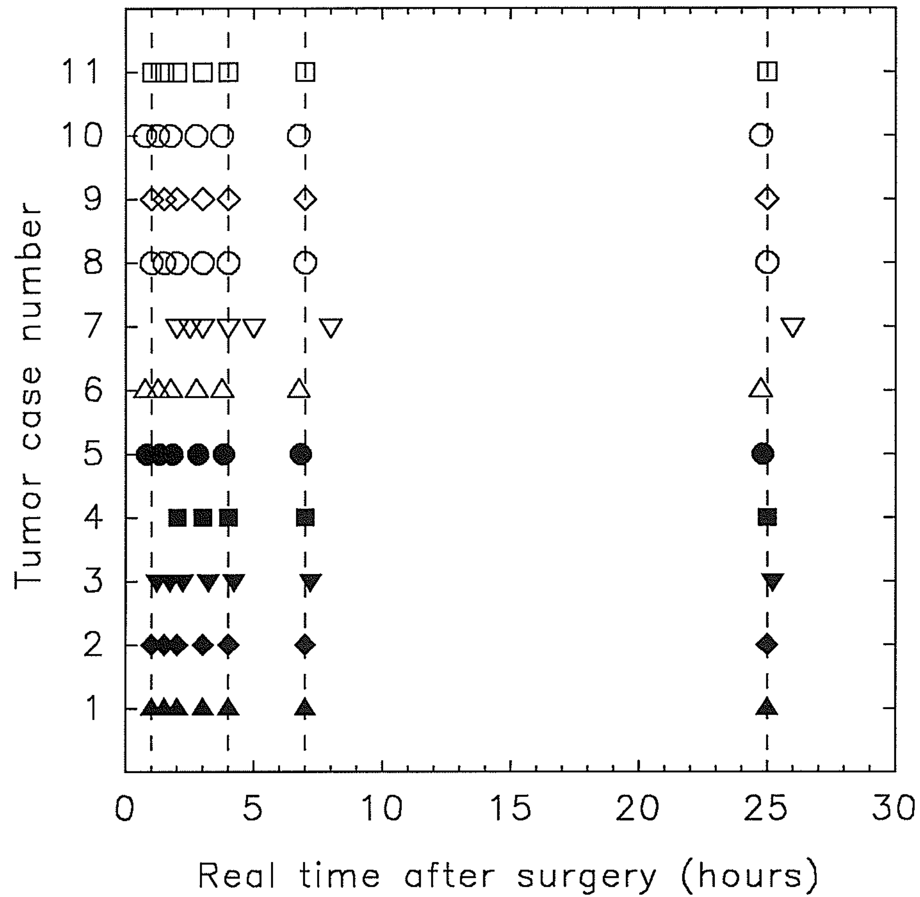


Figure 2.1 Time distribution for tumors. Distribution of time points for freezing of aliquots after samples from 11 tumors. The dotted lines correspond to relatively 'conserved' 'real time' points that were selected to represent 0, 3, 6 and 24 hours post surgery.

Construction of competitive RT-PCR fragment for c-*myc*

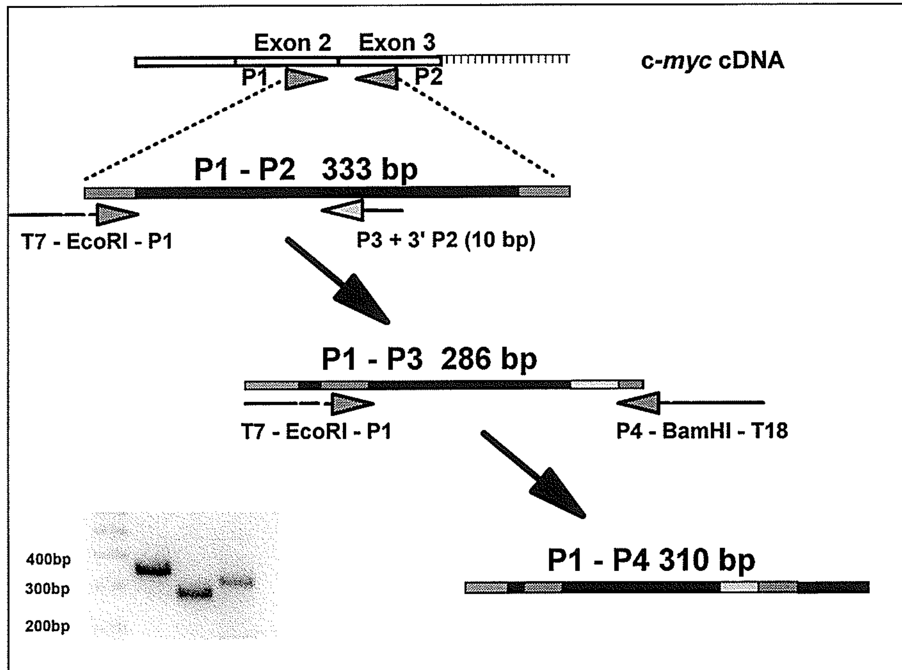


Figure 2.2 The strategy for creation of a synthetic standard to compete with the endogenous c-*myc* RNA transcript in a competitive RT-PCR reaction. The P1-P4 standard consists of a T7 polymerase promotor sequence at the 5' end to allow the generation of cRNA by in-vitro transcription, and a poly-T sequence at the 3' end to allow reverse transcription of the fragment in parallel with the endogenous c-*myc* RNA transcript. These 5' and 3' components are separated by an intervening sequence that is identical to 2/3 of the endogneous c-*myc* sequence that is amplified in parallel to ensure as far as is possible an equal amplification efficiency between the synthetic and endogenous transcripts.

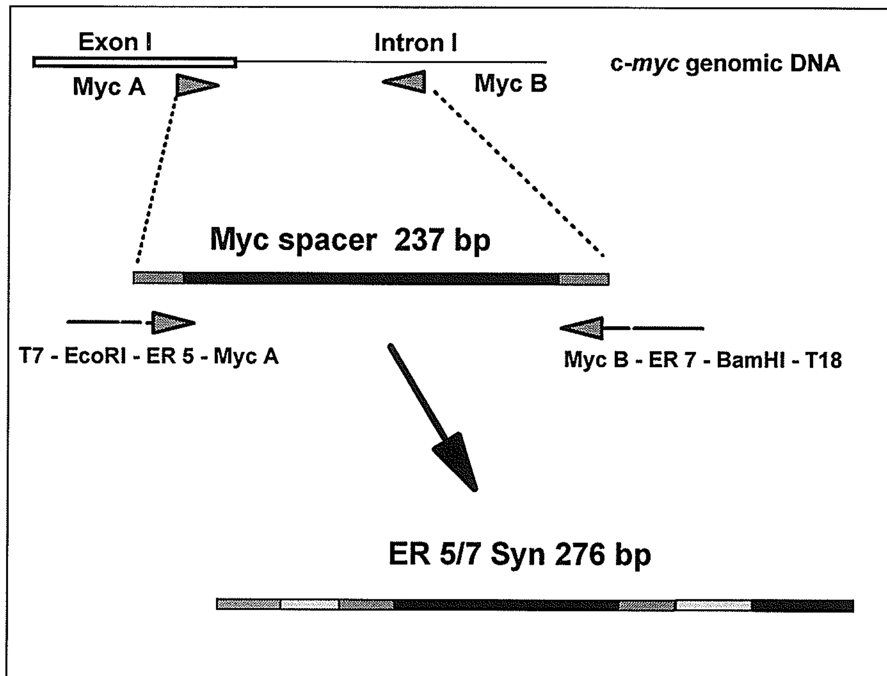


Figure 2.3 The strategy for creation of a synthetic standard to compete with the endogenous Estrogen Receptor mRNA transcript in a competitive RT-PCR reaction. The ER 5/7 Synthetic standard consists of a T7 polymerase promotor sequence at the 5' end to allow the generation of cRNA by in-vitro transcription, and a poly-T sequence at the 3' end to allow reverse transcription of the fragment in parallel with the endogenous ER RNA transcript. These 5' and 3' components are separated by an intervening spacer sequence that is derived from a sequence (1st intron from the c-*myc* gene) that is unrelated to the ER mRNA.

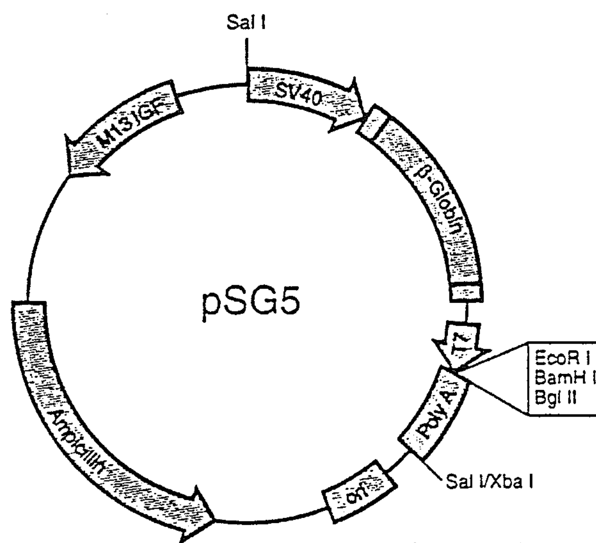


Figure 2.4 pSG5 vector for subcloning.

Quantitation of c-*myc* mRNA level

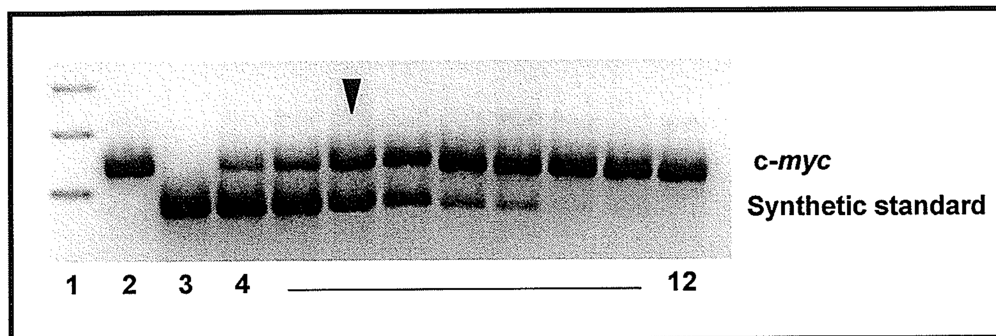


Figure 3.1 Competitive RT-PCR experiment to quantitate the level of c-***myc*** mRNA in MCF7 cell line showing that the c-***myc*** level is equivalent to 1.0 pg of synthetic standard. Arrow-head indicates the point of equivalence.

Lane 1 = size marker
Lane 2 = MCF7 RNA (50 ng)
Lane 3 = c-***myc*** synthetic standard (100 pg)
Lane 4 to lane 12 = MCF7 RNA (50 ng) combined with serial dilutions of competitive standard as follows: 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001 pg

Quantitation of Estrogen Receptor mRNA level

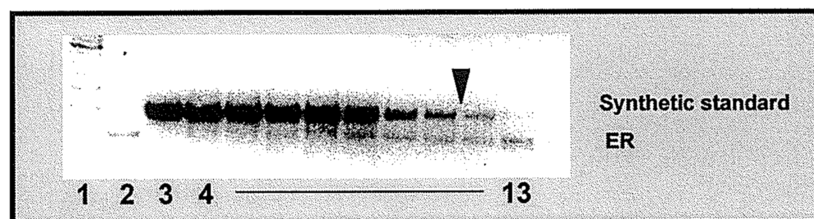


Figure 3.2 Competitive RT-PCR experiment to quantitate the level of Estrogen Receptor (ER) mRNA in T47D breast cell line showing that the ER level is equivalent to 2 pg of synthetic standard. Arrow-head indicates the point of equivalence.

Lane 1 = size marker

Lane 2 = T47D RNA (50 ng)

Lane 3 = ER synthetic standard (100 pg)

Lane 4 to lane 13 = T47D RNA (50 ng) combined with serial dilutions of competitive standard as follows:
100, 25, 10, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05 pg

Northern blot analysis with a panel of MCF7 cell line RNAs

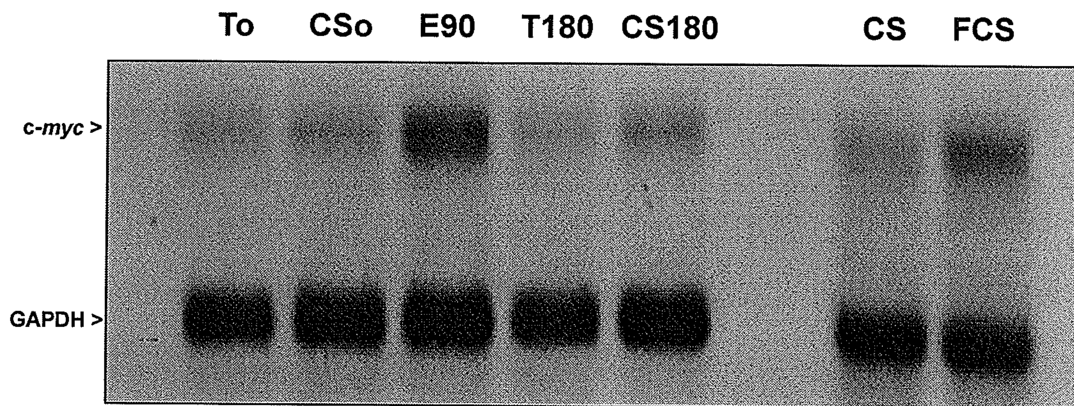


Figure 3.3 Quantitation of *c-myc* expression in a panel of MCF7 cell line RNA.
CSo=cells grown in charcoal treated fetal calf serum (FCS) at 0 hour, i.e. at the beginning of the experiment.
CS=cells grown in charcoal treated FCS.
CS180=cells grown in charcoal treated FCS at 180 minutes.
E90=cells grown in charcoal treated FCS rescued with estradiol (0.1 micro molar) at 90 minutes.
FCS=cells grown in normal FCS medium.
To=cells grown in charcoal treated FCS and tamoxifen (0.1 micro molar) at 0 hour.
T180=cells grown in charcoal treated FCS and treated with tamoxifen at 180 minutes.

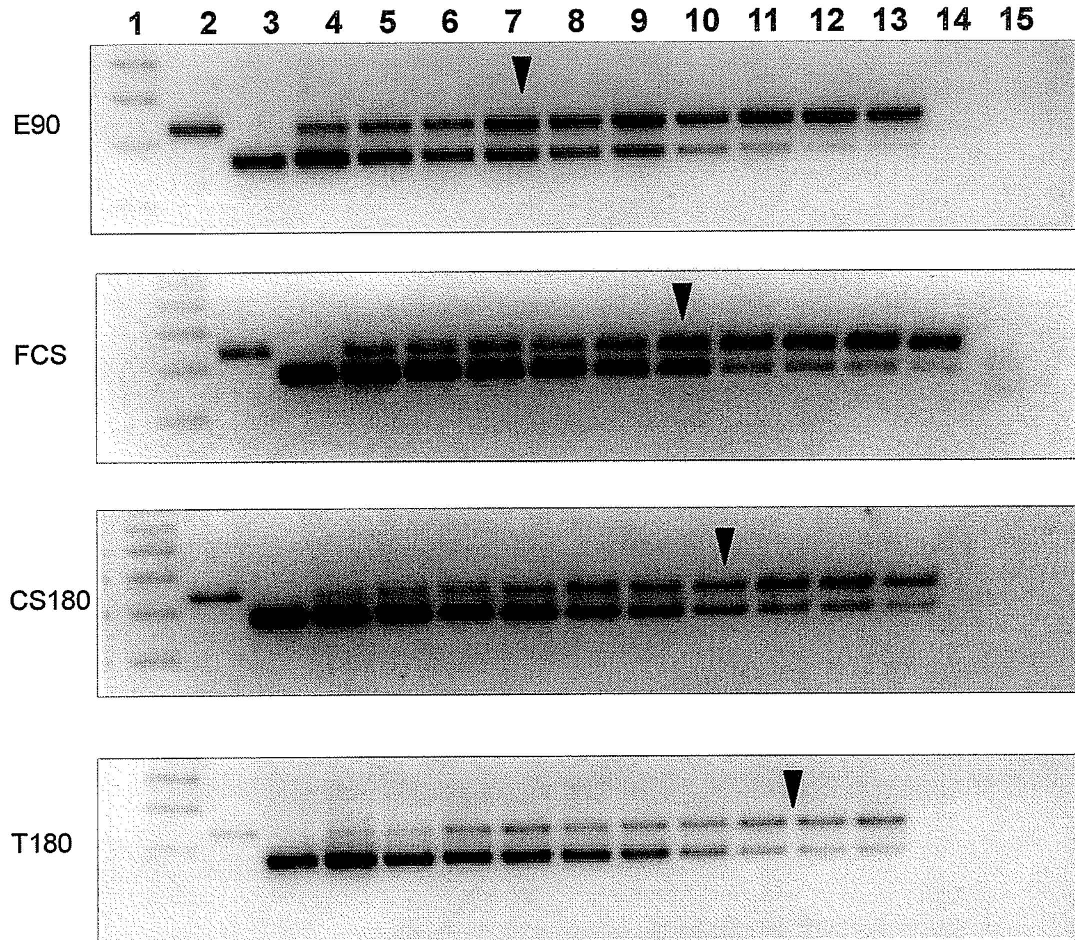
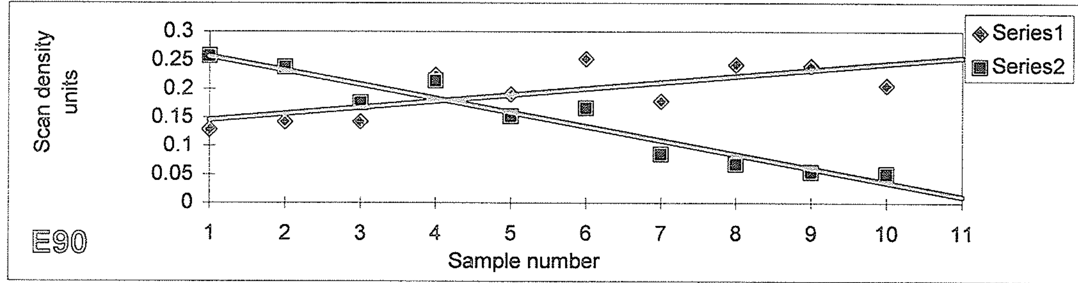
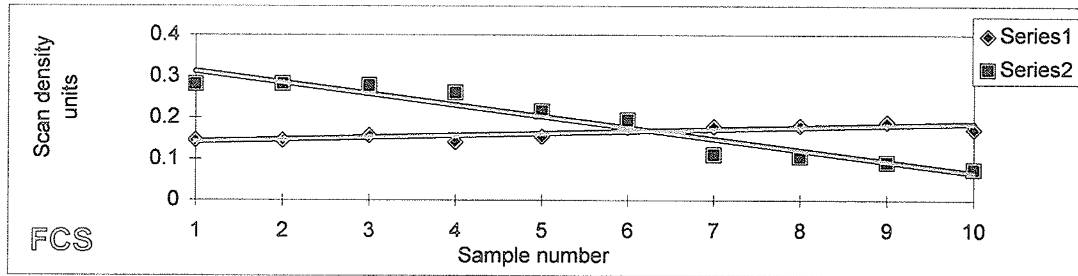
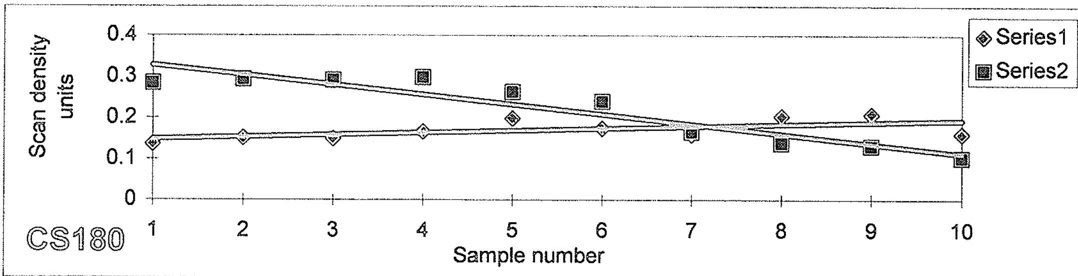


Figure 3.4 (a) Competitive RT-PCR experiment to quantitate the level of c-*myc* mRNA. Arrow-heads indicate point of equivalence.
E90=RNA from cells grown in charcoal treated fetal calf serum rescued with estradiol at 90 minutes.
FCS=RNA from cells grown in fetal calf serum.
CS180=RNA from cells grown in fetal calf serum treated with charcoal at 180 minutes.
T180=RNA from cells grown in charcoal treated fetal calf serum and treated with tamoxifen at 180 minutes.
Lane 1=Size marker
Lane 2= MCF7 RNA (50 ng)
Lane 3=Synthetic standard (100 pg)
Lane 4 to 13=MCF7 RNA (50 ng) combined with serial dilutions of competitive standard as follows: 40, 20, 10, 8, 6, 4, 2, 1, 0.8, 0.6 pg
Lane 14=RNA negative control
Lane 15=RTase negative control







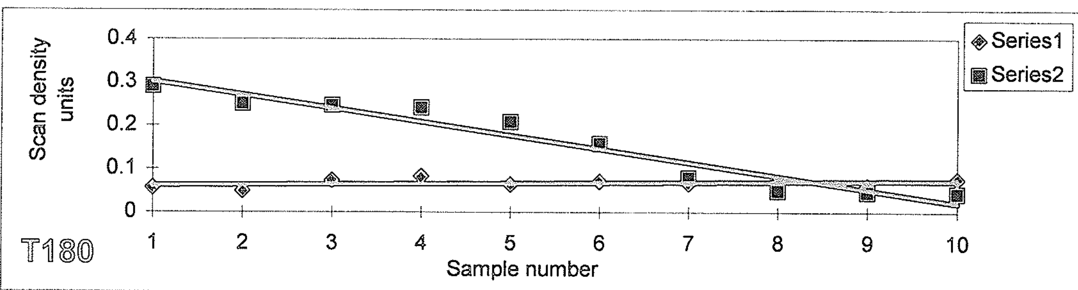


Figure 3.4 (b) Results of image analysis for RT-PCR assay for c-*myc* expression in MCF7 cell line RNAs shown in Figure 3.4 (a). Series 1=Endogenous RNA. Series 2=Synthetic standard RNA.

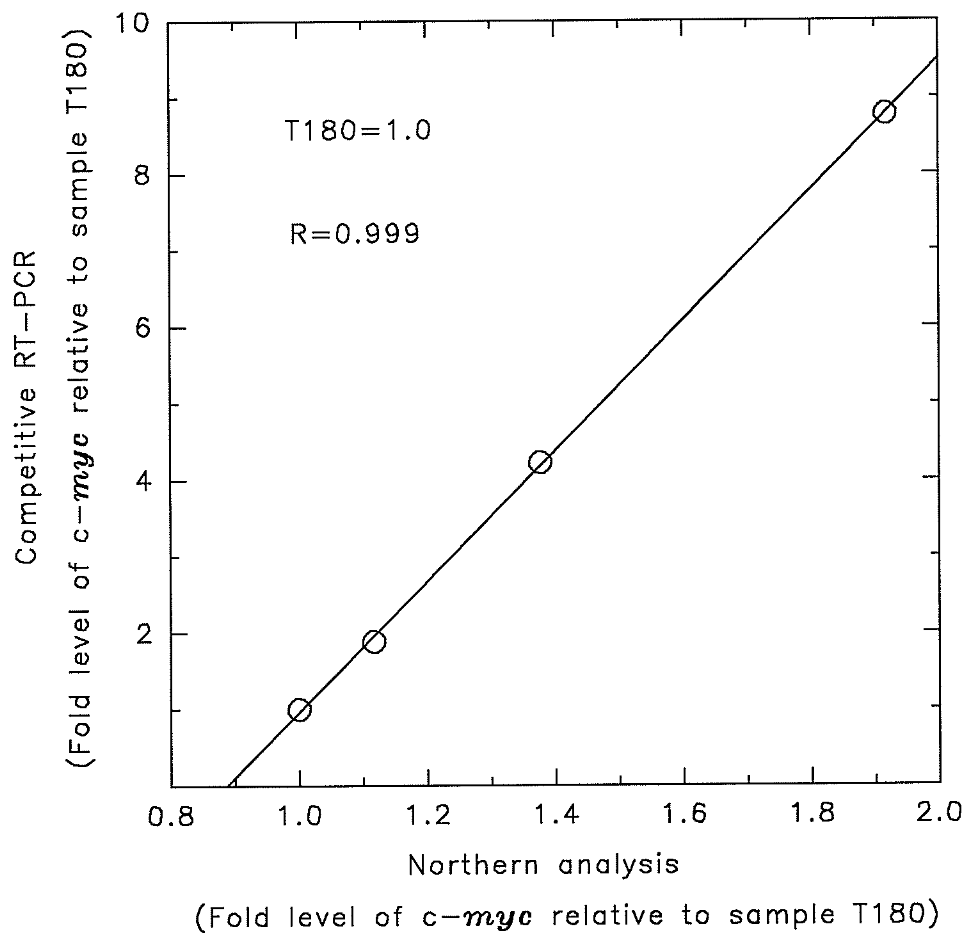


Figure 3.5 Comparison of competitive RT–PCR and Northern analysis. The values on both axes are relative to c–*myc* mRNA level in tamoxifen treated MCF7 cell line having lowest expression of c–*myc*.

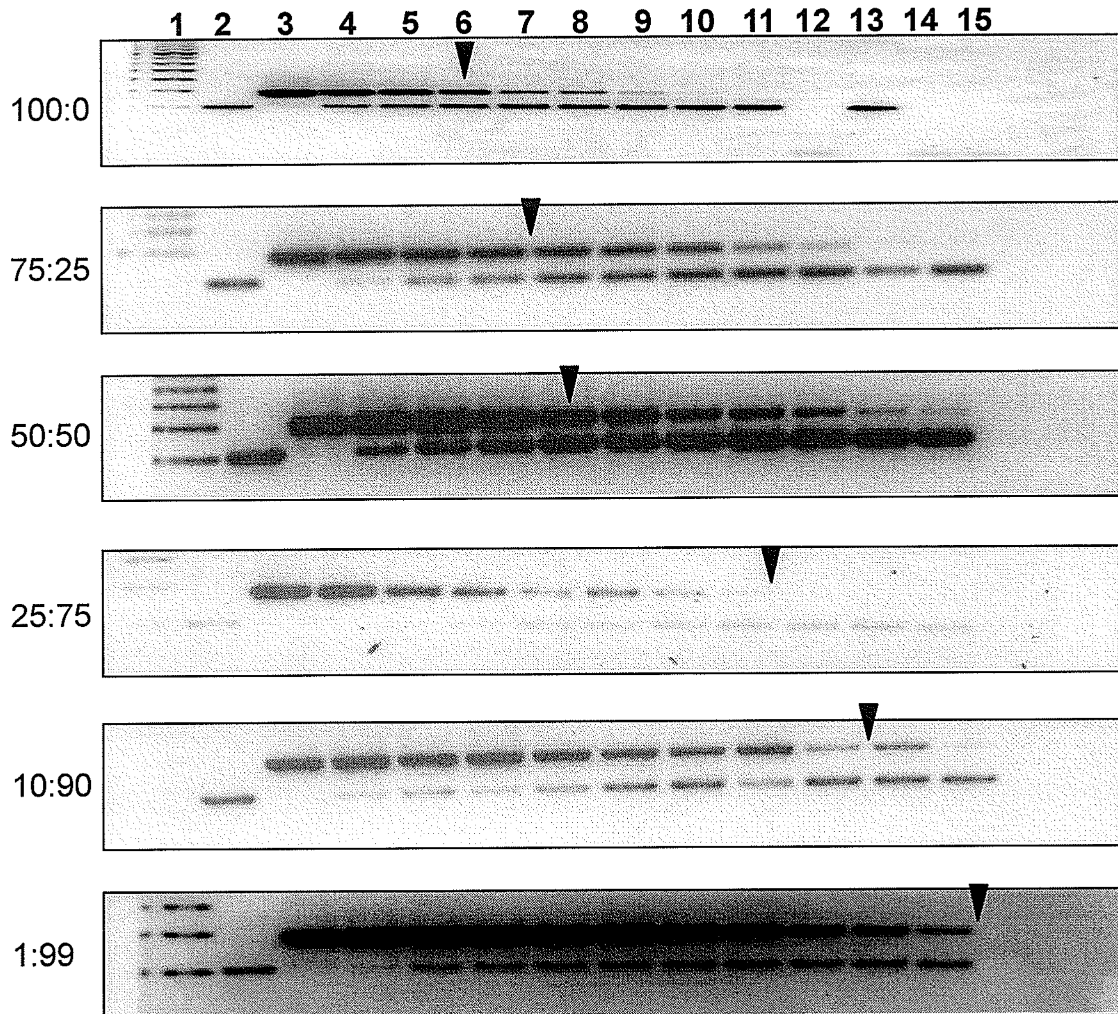


Figure 3.6 (a) Gel analysis of competitive RT-PCR experiment to quantitate the level of ER mRNA. Arrow-heads indicate point of equivalence.
Lane 1=Size marker
Lane 2=ER+/ER- RNA (50 ng)
Lane 3=Synthetic standard (100 pg)
Lane 4 to 13=ER+/ER- RNA (50 ng) combined with serial dilutions of competitive standard as follows: 100, 25, 10, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05 pg
Lane 14=RNA negative control
Lane 15-RTase negative control

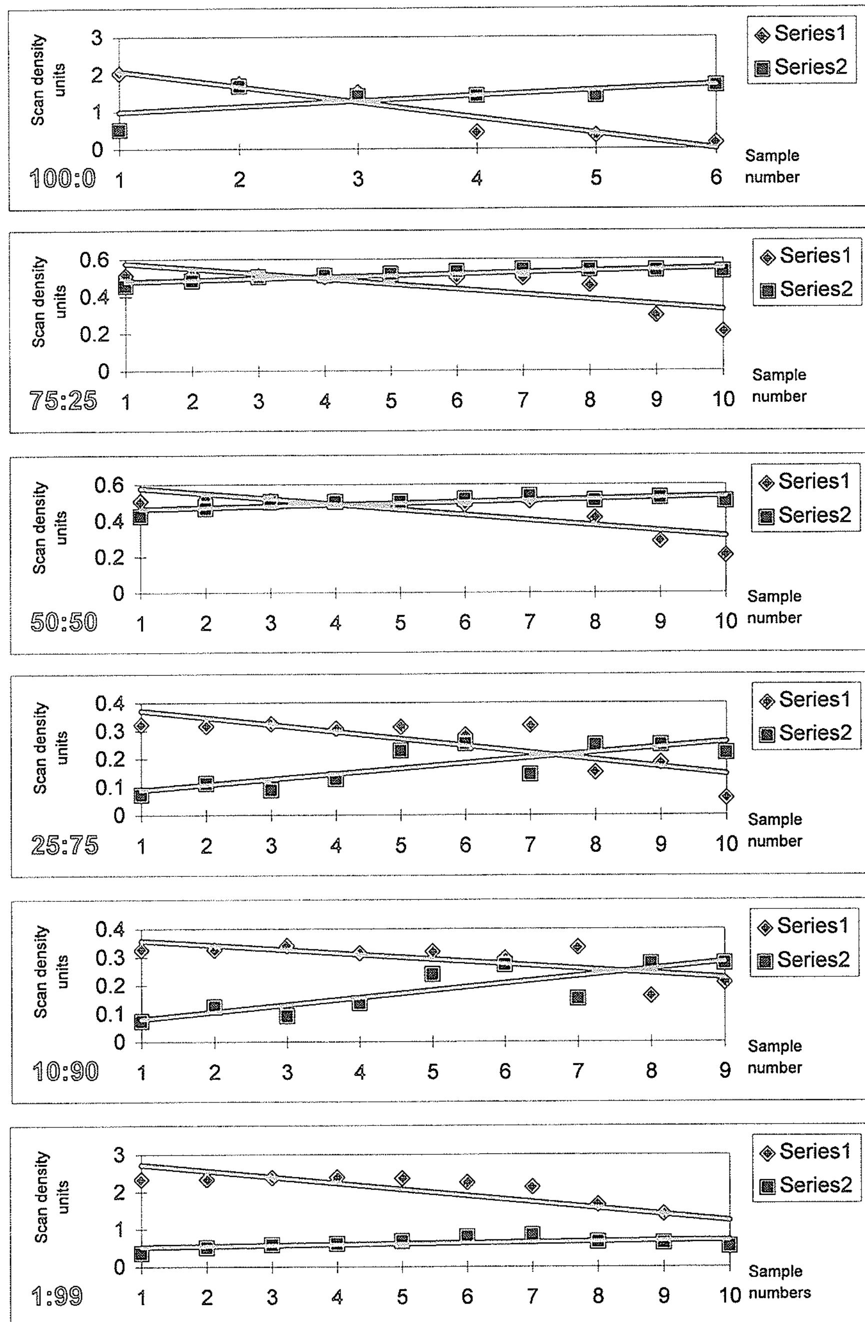


Figure 3.6 (b) Results of image analysis for RT-PCR assay for ER expression in a mixture of ER+ve/ER-ve RNAs. Note different x-axis range for various plots.

Series 1=Synthetic standard RNA.

Series 2=Endogenous RNA.

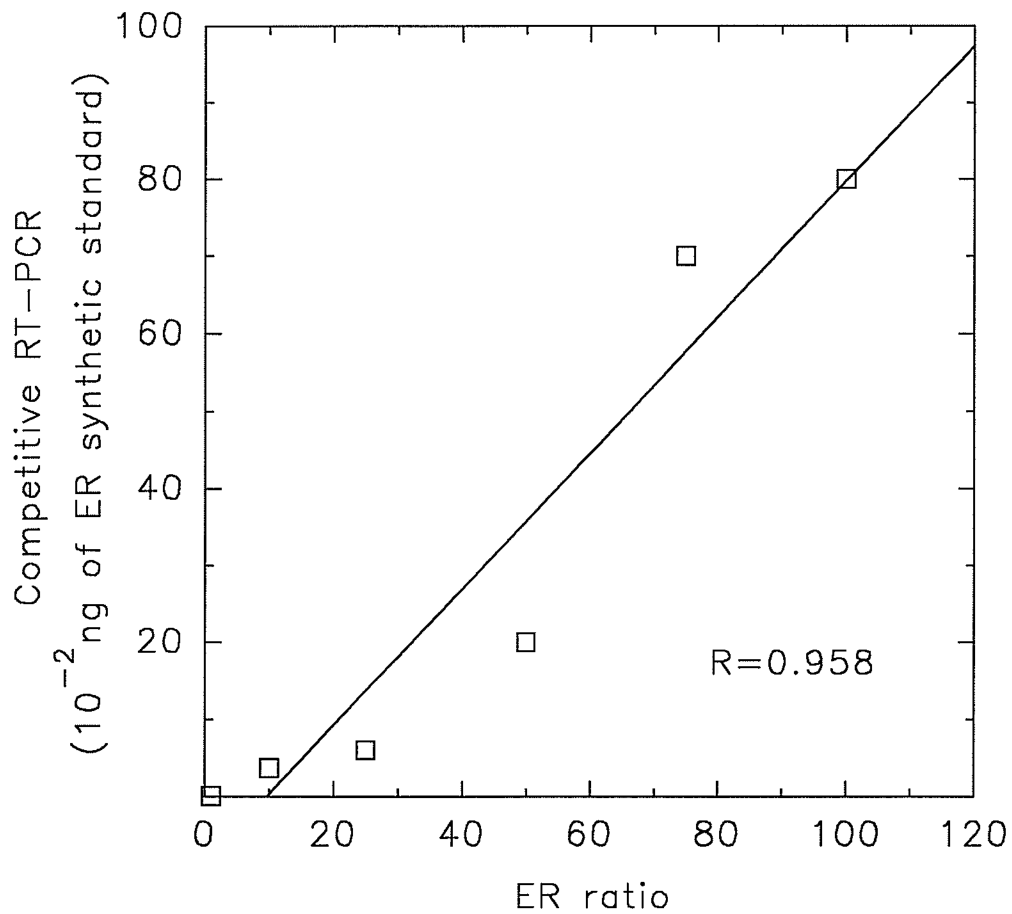


Figure 3.7 Validation test for the competitive RT−PCR assay for ER against RNA samples with unknown levels of ER.

Integrity of breast tumor RNA

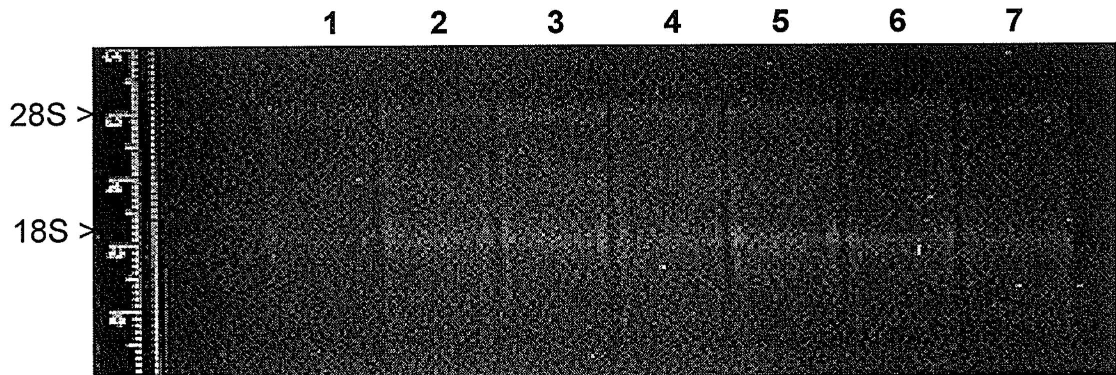


Figure 3.8 Result of Northern analysis done with 10 microgram RNA from tumor samples (case #2) collected and stored on ice before being frozen at -70 degree centigrade at specific project times.

Lane 1=sample frozen at 0 hour
Lane 2=sample frozen at 1/2 hour
Lane 3=sample frozen at 1 hour
Lane 4=sample frozen at 2 hour
Lane 5=sample frozen at 3 hour
Lane 6=sample frozen at 6 hour
Lane 7=sample frozen at 24 hour

Quantitation of c-*myc* mRNA in breast tumor samples

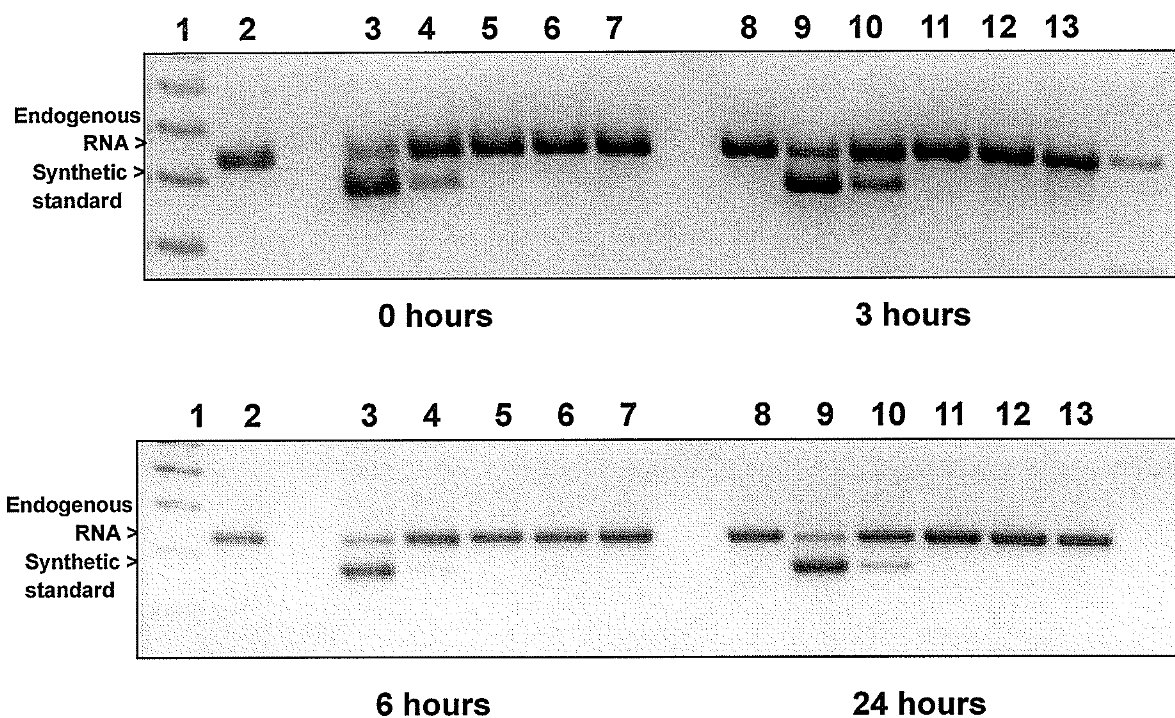


Figure 3.9 (a) Competitive RT-PCR experiment to quantitate the level of c-*myc* mRNA in breast tumor (case #8).
Lane 1=size marker
Lane 2 and 8=endogenous RNA (50 ng)
Lane 3 to 7 and 9 to 13=tumor RNA (50 ng) combined with serial dilutions of competitive RNA standard as follows: 0.01, 0.001, 0.0001, 0.00001, 0.000001 pg

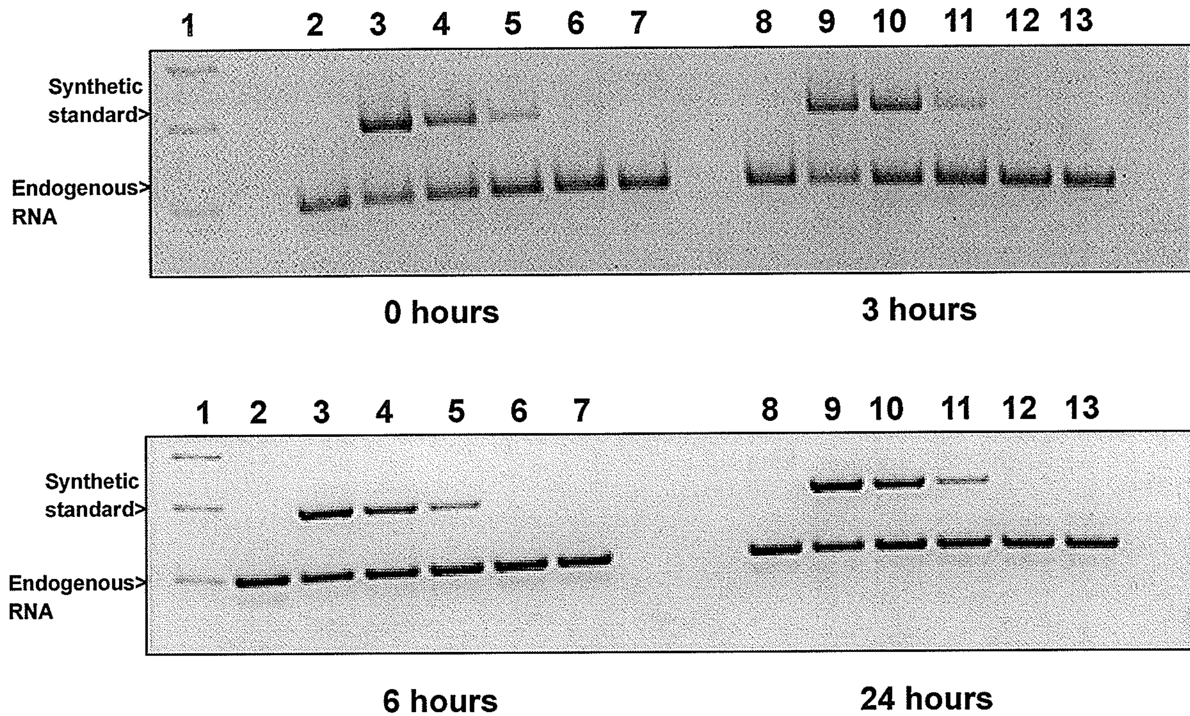


Figure 3.9 (b) Competitive RT-PCR experiment to quantitate the level of ER mRNA in breast tumor (case #4).
Lane 1=size marker
Lane 2=endogenous RNA (50 ng)
Lane 3 to 5=tumor RNA (50 ng) combined with serial dilutions of competitive standard as follows: 0.1, 0.01, 0.001 pg
Lane 6 and 12=RNA -ve control
Lane 7 and 13=RTase -ve control

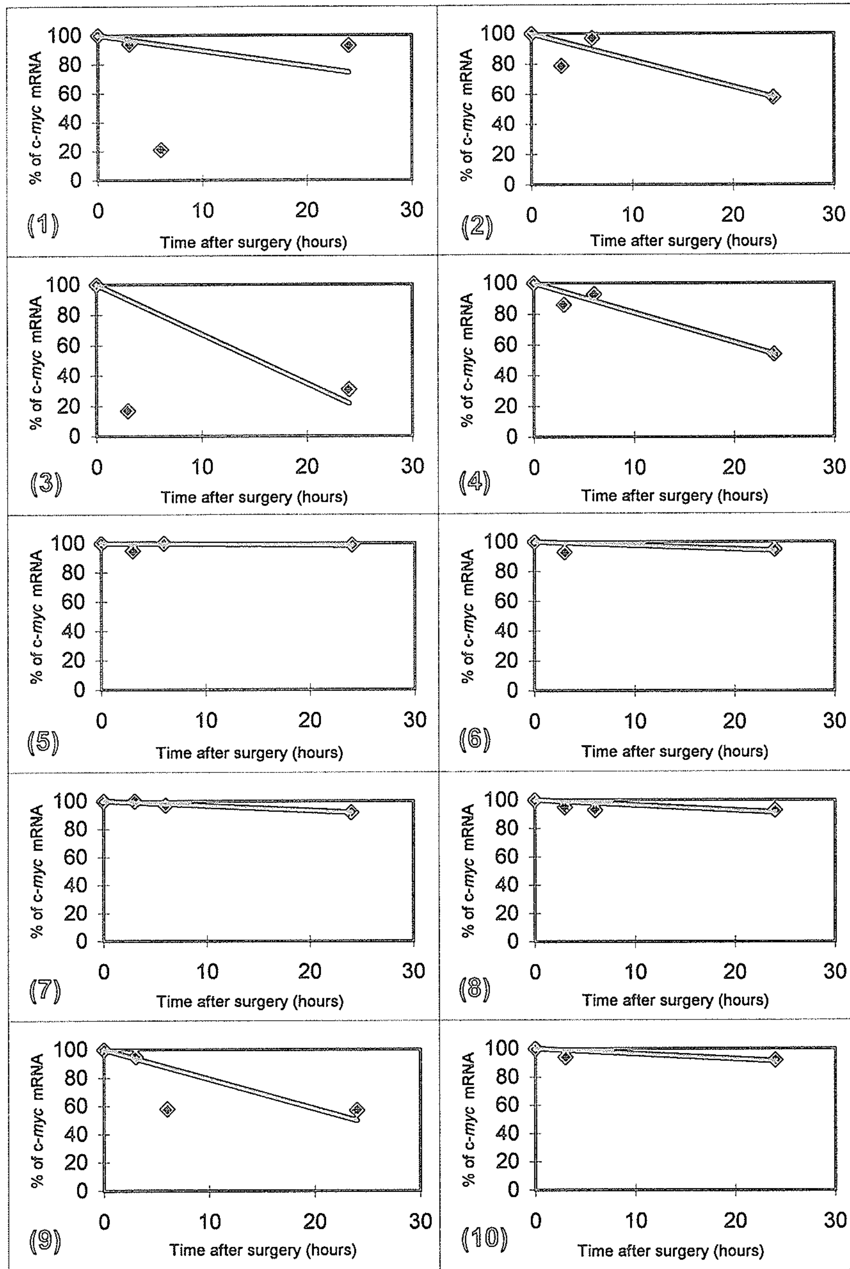


Figure 3.10 Effect of tumor collection time on c-*myc* mRNA level in surgically resected breast cancer specimens. Tumor number indicated in parentheses of respective plot.

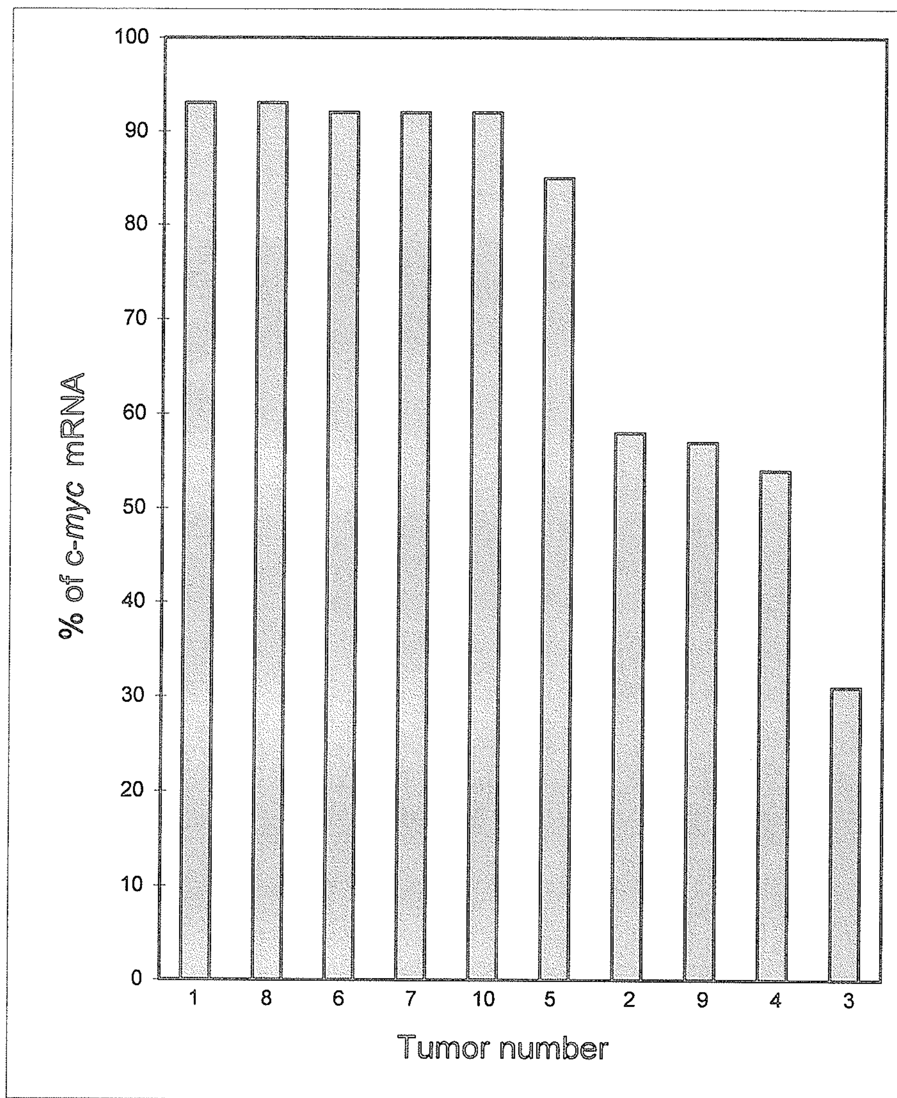
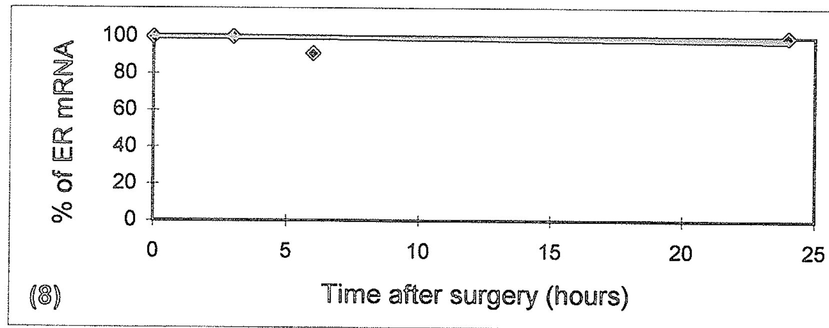
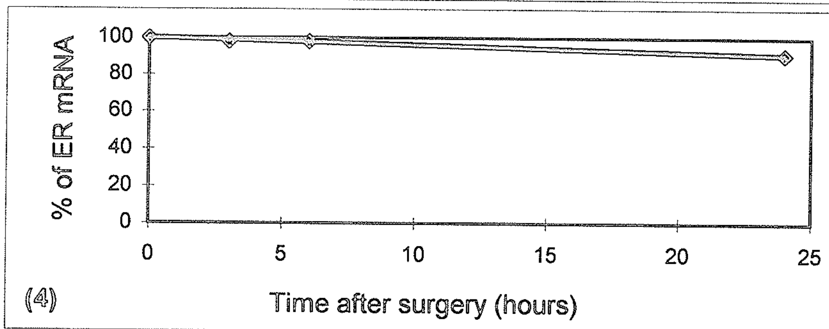
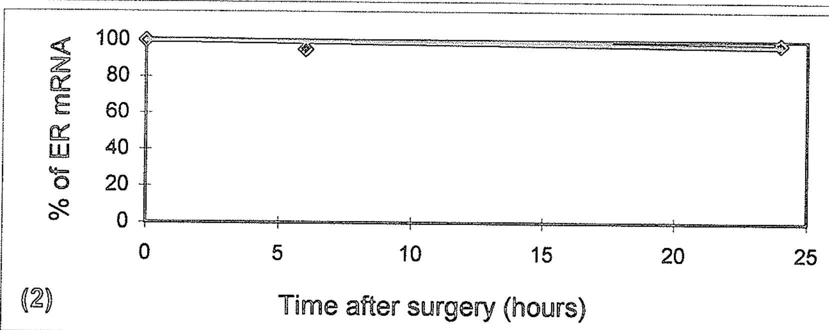


Figure 3.11 Change in c-*myc* mRNA level relative to time zero over a project time of 24 hours.







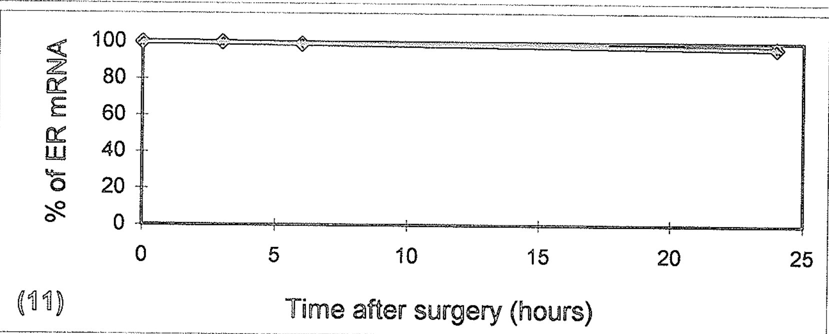


Figure 3.12 Effect of tumor collection time on ER mRNA level in surgically resected breast cancer specimens. Tumor number indicated in parentheses of respective plot.

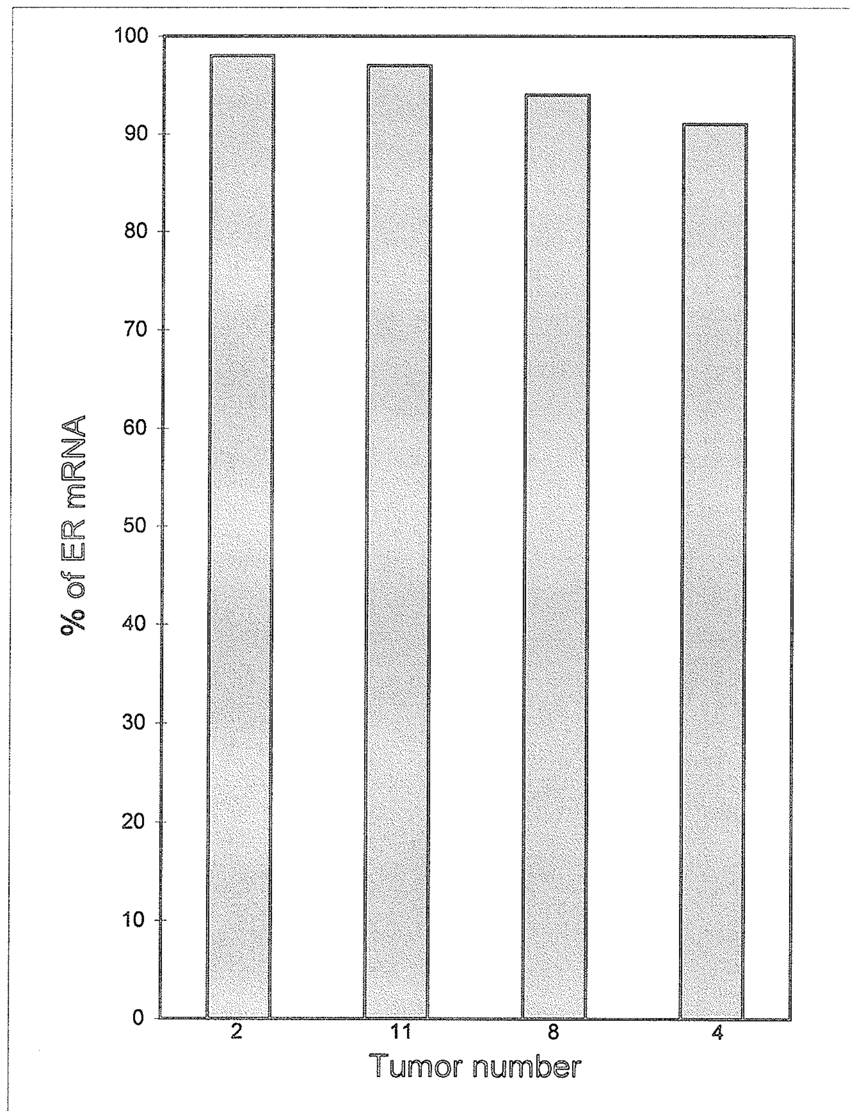


Figure 3.13 Change in ER mRNA level relative to time zero over a project time of 24 hours.