The Analysis of Novel Gene Targets in Breast Cancer in Women ≤35 years

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Abstract

Breast cancer presenting in women aged ≤ 35 years has a more aggressive behaviour, and thus poorer prognosis, which is thought to be due to differences in the tumour biology of these tumours compared to those from older women.

The aim of this study was to examine the expression of 9 novel gene targets (A-kinase anchor protein-1 (AKAP1), Acidic protein rich in leucines (APRIL), CCAAT enhancer binding protein alpha (C/EBPα), Damage-specific DNA binding protein 2 (DDB2), Granulin, Nuclear Receptor Coactivator 3 (NCOA3), Retinoic acid receptor Responder 3 (RARRES3), Retinoblastoma binding protein 4 (RBBP4), and Transforming Growth Factor beta Induced (TGFβI) identified previously by a cDNA microarray in breast cancers and female controls by RT-qPCR, western blotting, and immunohistochemistry.

Six breast cell lines, 9 samples of organoids from reduction mammoplasty tissues, and 35 tumour tissues (20 cases >35 years, 15 cases ≤35 years in age) were analysed for the expression of the nine target genes using real time quantitative RT-PCR. Of the nine target genes investigated, five showed differences between normal and cancers ≤35, or between breast cancers ≤35 and those >35 years. NCOA3 and RARRES3 showed elevated levels of mRNA in breast cancers ≤35 years compared to those >35 years ($p= 0.001$ and $p=0.002$ respectively). Compared to the normal breast, TGFβI showed a reduced level of mRNA expression in both younger and older cases ($p= 0.026$ and $p=0.001$ respectively), while DDB2 and C-EBPα showed a reduced level of mRNA expression in younger group only ($p= 0.002$ and $p=0.001$ respectively).

NCOA3 protein expression examination using western blotting found high levels in the ER+ve cell lines MCF-7, ZR-75-1 and T47-D with a weak expression in ER−ve cell lines HBL-100 and MDA-MB-468. RARRES3 protein expression was found in 4 breast cell lines (HBL-100, MDA-MB-468, MCF-7, and ZR-75-1). IHC found expression of NCOA3 in younger and older tumours including ER+ve and ER−ve cases.

This study identifies NCAO3 and RARRES3 as potential markers for breast cancers in younger women, but the data need confirmation in a larger series of cases.

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ADH	Atypical ductal hyperplasia
AJ	Alec Jeffrey's
AKAP1	A-kinase anchor protein 1
AIB1	Amplified in breast cancer 1
ALH	Atypical lobular hyperplasia
AMV-RT	Avian myeloblastosis reverse transcriptase
ANOVA	Analysis of variance
AP-1	Activator protein-1
APRIL	Acidic protein rich in leucines
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated
BRCA1/2	Breast cancer susceptibility gene ½
BSA	Bovine serum albumin
CAF-1	Chromatin assembly factor 1
CCAAT EBPα	CCAAT enhancer binding protein alpha
CDKs	Cyclin dependent kinases
cDNA	complementary deoxyribonucleic acid
CGH	Comparative genomic hybridisation
CHEK2	Checkpoint-like protein 2
CPDs	Cyclobutane pyrimidine dimers
Ct	cycle threshold
CYP	Cytochrome P450
DCIS	Ductal carcinoma *in situ*
DDB2	Damage-specific DNA binding protein 2
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
ER	Eoestrogen receptor
ESTs	Expressed sequence tags
EtOH	Ethanol
FISH	Fluorescence *in situ* hybridisation
FFPE	Formalin-fixed paraffin-embedded
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEP	Gene expression profile
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HDACs	Histone deactylases
H&E	Haematoxylin and eosin
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HER-2	Human epidermal growth factor receptor 2
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IDC	Infiltrating ductal carcinoma

ILC	Infiltrating lobular carcinoma
IMS	Industrial methylated spirits
LCIS	Lobular carcinoma *in situ*
LOH	Loss of heterozygosity
MTHFR	5,10-Methylenetetrahydrofolate reductase
M	Molar
$MgCL_2$	Magnesium Chloride
Mg	milligram
mM	Micromolar
mL	Millilitre
mRNA	messenger ribonucleic acid
NCOA3	Nuclear receptor coactivator 3
NER	Nucleotide excision repair
NFκB	Nuclear factor kappa B
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Pleural infusion
PGBD	Porphobilinogen deaminase
PgR	Progesterone receptor
PK	Proteinase K
PKA	Protein kinase
PKC	Phospholipid-dependent kinase
PLC	Pleomorphic variant of lobular carcinoma
pM	picoMolar
PTEN	Phosphatase and tensin homolog gene
RARRES3	Retinoic acid receptor responder 3
Rb	Retinoblastoma
RBBP4	Retinoblastoma binding protein 4
RIG-1	Retinoid-inducible gene 1
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAM	Significance analysis of microarrays
SD	Standard deviation
SEM	Standard error of the mean
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STAT	Signal transducer and activator of transcription
TBS	Tris-HCL buffered saline
TE	Tris EDTA
TFRC	Transferrin receptor
TGFβI	Transforming growth factor *beta* induced
TIG-3	Tazarotene-induced gene 3
Wt	Wild-type
µg	microgram
µL	microlitre
µM	micromolar

Chapter 1. Introduction

1.1 Introduction to Breast Cancer

Breast cancer is the most common cancer in women worldwide (Parkin, 2001). In the UK and Ireland, breast cancer accounted for almost 1 in 3 of all female malignant cancers in the 1990s, when the number of new cases averaged 44,000 each year (National Statistics, 2007). The surge in the numbers of breast cancers diagnosed is probably due to improved detection by breast mammography screening programmes as well as the fact that women are living longer.

Incidence and mortality both vary around the world (Parkin & Muir, 1992), with a high incidence in the West and lower incidence in the Far East. Incidence is increasing in many parts of the world, including the USA, Canada, and Europe as well as the Far East. Breast cancer has also been the most common cause of cancer death in women, responsible for almost 1 in 5 of all cancer deaths, and 5 per cent of all deaths in women in the 1990s (National Statistics, 2007). Risk for breast cancer is higher for post menopausal women, with 80% off all cases diagnosed in those over 50 years old. Survival rates for breast cancer have improved over the past 20 years. In 1989, 15,625 women died from the disease compared to 12,417 in 2005. The estimated five-year survival rate for women diagnosed in England and Wales in the 1970s was around 50%, whereas now it's closer to 80% (National Statistics, 2007). This fall in the number of deaths can be attributed to earlier detection due to an increased awareness among women, widespread screening programmes, and improved treatment.

1.2 Risk factors

The aetiology of breast cancer is still poorly understood with known risk factors explaining only a minority of cases. The identification of these risk factors, both environmental and genetic, has been critical in the search for improvements in diagnosis and prevention, even if they do not explain the underlying mechanisms. General risk factors for breast cancer include all of the following: increasing age, geographic location, socioeconomic status, reproductive factors i.e. late age of menopause, early age of menarche, nulliparity, and older age at first birth, exogenous hormones, lifestyle risk factors (diet, obesity, alcohol, physical activity), as

well as genetic factors (family history and high- and low- penetrance breast cancer susceptibility genes).

1.2.1 Age, ethnicity, location

Cancer is considered a disease of aging, as evidenced by the rising incidence with increasing age. Incidence of breast cancer is less than 2 cases per 100,000 in women aged less than 25, over 100 cases per 100,000 in women at age 40, and over 400 cases per 100,000 in women aged 80 and over (Ries *et al.* 2004). In the USA, incidences are 20-40% higher in white women than in African-American (Garfinkel *et al.*, 1994), except in younger age groups where rates are higher in African-Americans (Bernstein *et al.*, 2003). There is a definite trend for breast cancer to be more common in areas such as North America, and Northern Europe, while areas in the Far East such as Japan have much lower rates of the disease (Parkin *et al.*, 1999).

1.2.2 Family history

Having a family history can increase a woman's chance of developing breast cancer. Epidemiological studies have shown that ~12% of breast cancer patients have one or more first-degree relatives affected with the disease. A large study found that women with a first-degree relative with the disease have a 2-fold increase in risk of breast cancer (CGHFBC, 2002). The risk of breast cancer increases with the number of affected relatives and is greater for women with a relative affected at a younger age, bilateral disease or a history of benign disease (McPherson *et al.*, 2000).

1.2.3 Breast density, benign breast disease, and radiation

Women with more than 75% increased breast density on mammography have around 5-fold increase in the occurrence of breast cancer over a woman with 5% increased breast density (Byrne *et al.*, 2001). Pre- and postmenopausal nulliparous women tend to be thinner, and in general, have an increased breast density (Biglia *et al.*, 2004), and therefore have an increase risk for developing breast cancer.

A history of benign breast disease is known to increase the risk of developing breast cancer. Those with severe atypical epithelial hyperplasia have around 4-fold increased risk of

developing breast cancer, while those who also have a family history of breast cancer have a nine-fold increase in risk (McPherson *et al.*, 2000).

Exposure to ionising radiation is known to increase the risk of developing breast cancer. This theory has been supported by the high incidence among women exposed to Hiroshima and Nagasaki nuclear explosions (Tokunaga *et al.*, 1987).

1.2.4 Hormones

Lifetime exposure to endogenous sex hormones is determined by many factors, such as age at menarche, age at first pregnancy, number of births, and age at menopause, all of which have been examined for their influence on breast cancer risk. An early age at menarche appears to increase breast cancer risk by 10-20% (Berkey *et al.*, 1999), while a later onset of menopause increases the risk by ~3% (Collaborative Group on Hormonal Factors in Breast Cancer, 2001). Pregnancy at a younger age in particular, as well as breastfeeding is believed to lower the risk of breast cancer. Obesity has been found to result in a higher risk for breast cancer (Huang *et al.*, 1997) (since oestrogen is found at high levels in adipose tissue), whereas physical activity during youth decreases breast cancer risk by 20% (Lagerros *et al.*, 2004). The decrease in risk found with increased physical activity is thought to be due its effect on delaying onset of menarche and modifying the bioavailable hormone levels. In postmenopausal women, the higher risk of developing breast cancer associated with high bone density is attributed to the fact that oestrogens are known to assist in bone mass maintenance (Kuller *et al.*, 1997).

Exposure to exogenous hormones such as oral contraceptives and hormone replacement therapy result in a small increased risk of developing breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 2001).

1.2.5 Diet and lifestyle

Alcohol consumption has been associated with an increased risk for breast cancer development (Smith-Warner *et al.*, 1998). Breast cancer incidence is higher among women from urban areas than in those from rural regions (Wronkowski *et al.*, 1993), while those from a lower socio-economic backgrounds had a decreased risk of developing breast cancer (Lawson, 1999).

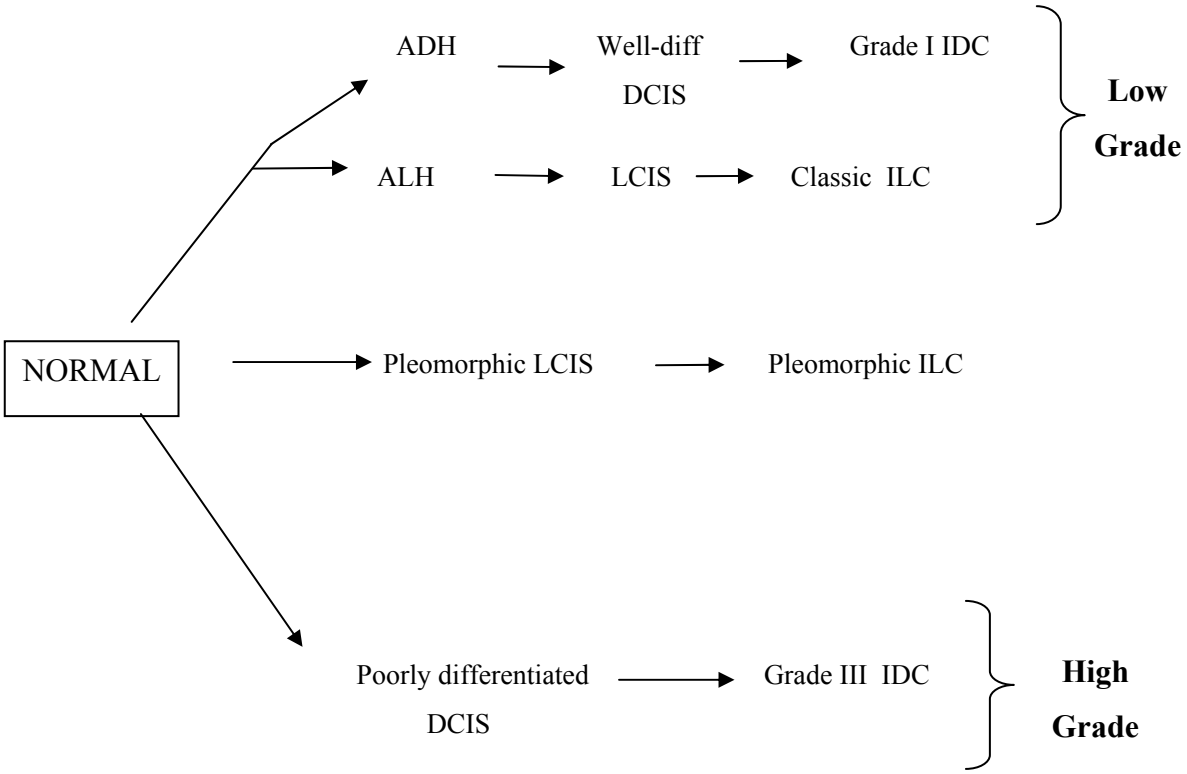
1.3 Development of breast cancer

Historically breast cancer development has been seen to be a multi-step process including a progression from normal, to hyperplasia, carcinoma *in situ*, invasive carcinoma, and metastasis. While the basic concepts of the morphology of these breast cancer precursor lesions remains legitimate, advances in immunohistochemistry and molecular genetics have changed the way the multi-step process is interpreted. It is now clear that the process of breast cancer development comprises two different arms with their own pathways (Simpson *et al.*, 2005).

The first refers to the progression of atypical ductal/lobular hyperplasia to well-differentiated ductal/lobular carcinoma *in situ* (DCIS/LCIS), which in turn progresses to grade I invasive ductal/lobular carcinoma (IDC/ILC) ('low-grade arm') (see Figure 1.1). The second involves the development of grade III IDC from poorly differentiated DCIS ('high-grade arm'). 'Low-grade arm' cancers tend to be ER and PgR positive, of low nuclear grade, negative for HER-2 and basal markers, and have low genetic instability with 16q loss. 'High-grade arm' cancers tend to show more nuclear atypia, are normally ER and PgR negative, may be positive for HER2, and have numerous genomic alterations, such as loss of 8p, 11q, 13q, 14q, gain of 1q, 5p, 8q, 17q, and amplifications on 6q22, 8q22, 11q13, 17q12, 17q22-24, and 20q13 (Buerger *et al.*, 1999; Reis-Filho & Lakhani, 2003; Roylance *et al.*, 1999).

Lobular carcinoma *in situ* (LCIS) and invasive lobular carcinoma (ILC) belong to the 'low-grade arm' (Lu *et al.*, 1998). However, they are dissimilar to the well-differentiated DCIS and grade I IDC as they do not express E-cadherin (Cleton-Jansen, 2002). Pleomorphic variant of lobular carcinoma (PLC) shares morphological features with both classic ILC and grade III IDC, with loss of 16q and no E-cadherin expression, although, it can show HER-2 overexpression. Therefore, the boundaries between the 'low-grade arm' and the high-grade arm' are slightly distorted, demonstrating that the molecular pathways in breast cancer carcinogenesis are extremely complex.

Figure 1.1 Model for breast cancer progression



ADH: atypical ductal hyperplasia; ALH: atypical lobular hyperplasia; Well-diff: well differentiated; DCIS: ductal carcinoma *in situ*; IDC: infiltrating ductal carcinoma; ILC: infiltrating lobular carcinoma; LCIS: lobular carcinoma *in situ*;

1.4 Breast cancer susceptibility genes

Breast carcinogenesis is a multi-step process, with each step thought to involve one or more distinct genetic mutations (see section 1.3). Hereditary breast cancer is characterised by an inherited susceptibility to breast cancer due to germline mutations in high-risk breast cancer susceptibility genes such as BRCA1, BRCA2, p53, E-cadherin, LKB1, and PTEN, or low-risk genes such as ATM, and CHEK2, whereas sporadic breast cancer arises from a series of somatic mutations these and other cancers (Wooster & Weber, 2003).

1.4.1 BRCA1 and BRCA2

Breast cancer susceptibility gene 1 (BRCA1), and breast cancer susceptibility gene 2 (BRCA2), are tumour suppressor genes located on chromosome 17q21 and 13q12-13 respectively (Hall *et al.*, 1990; Wooster *et al.*, 1994). Both genes code for proteins whose primary function is to maintain genomic integrity. BRCA1 and BRCA2 mutations account for 5-10% of all breast cancer cases and approximately 80% of all hereditary breast cancer cases (Rebbeck *et al.*, 1996). The cumulative risk of breast cancer at age 70 years with a mutation in BRCA1 or BRCA2 is 85% and 84% respectively and 63% and 27% respectively for ovarian cancer (Ford *et al.*, 1998). Loss of heterozygosity (LOH) in the BRCA1 gene is often seen in hereditary breast cancers, and is the most common cause of gene inactivation (Osorio *et al.*, 2002).

Germline mutations of BRCA2 are associated with approximately 76% of breast cancer families in which both females and males are affected, compared to 32% in families where women only are affected (Ford *et al.*, 1998). The prevalence of carriers of mutations in BRCA1 and BRCA2 is approximately one in 1,000 and one in 750 respectively (Antoniou *et al.*, 2002).

1.4.2 p53

The tumour suppressor gene p53 is located on chromosome 17p13.1 and encodes a protein involved in important cellular processes including cell cycle, apoptosis, and DNA-repair. Germline mutations in p53 are responsible for Li-Fraumeni syndrome, a disease associated with breast cancer (1% of all breast cancers) (Srivastava *et al.*, 1990). Other hereditary breast cancers may be due to mutations in genes coding for p53 modulator proteins. A significant proportion of these cancers have been associated with mutations of *BRCA1*.

BRCA1 may interact with p53 and has been viewed as a 'scaffold' for p53 response (Hohenstein & Giles, 2003). BRCA1 tumours often harbour p53 mutations, but it is not yet known if this infers the need for p53 inactivation for the development of BRCA1 tumours to occur, or if the loss of BRCA1-associated DNA repair properties may explain, in some way, the high frequency of p53 mutations (Lacroix & Leclercq, 2005).

1.4.3 PTEN

The phosphatase and tensin homolog gene (PTEN) tumour suppressor located on chromosome 10q23 is mutated in Cowden syndrome, a disorder characterised by multiple benign tumours. PTEN mutations found in Cowden syndrome have been linked with cancer (Marsh *et al.*, 1999), and are associated with a 25-50% lifetime breast cancer risk. It accounts for a small proportion (<1%) of hereditary breast cancers.

1.4.4 LKB1

The LKB1 gene is located on chromosome 19p13.3 and encodes a tumour suppressor protein. Mutations result in Peutz-Jegher syndrome, a rare syndrome characterised by gastrointestinal polyps and freckles on the skin. Patients with Peutz-Jeghers syndrome have a 29-54% chance of developing breast cancer by age 65 (Lim *et al.*, 2003).

1.4.5 ATM

The ATM gene located on chromosome 11q22-23 encodes a protein which plays an important role in detecting the presence of DNA double-strand breaks. Carriers of ATM mutations suffer from Ataxia telangiectasia (AT), a disease that incurs an increased susceptibility to cancers. Studies of individuals with AT have suggested that female relatives heterozygous for an ATM mutation have a 2-5 fold increase in risk of breast cancer (Swift *et al.*, 1987). The risk for developing breast cancer in ATM carriers is about 11% at age 50, and 30% by age 70 (Easton, 1994).

1.4.6 CHEK2

CHEK2 is a G2 checkpoint kinase that plays a central role in DNA repair. Mutations in CHEK2 were first identified in a family with Li-Fraumeni syndrome, and later found in several breast cancer samples (Vahteristo *et al.*, 2001). It was later found that the

CHEK2*1100delC mutation occurs in over 5% of breast cancers from women with a family history of the disease but with no BRCA1 or BRCA2 mutations (Meijers-Heijboer *et al.*, 2002).

1.4.7 E-cadherin

The E-cadherin gene is located on chromosome 16q22.1, and encodes a cell-cell adhesion protein, that plays a central role in the maintenance of cell differentiation and architecture of epithelial tissues. Mutations in this gene are associated with hereditary diffuse gastric cancer syndrome. Patients with this syndrome, have a 20-40% risk of developing breast cancer (Pharoah *et al.*, 2001).

1.4.8 Polymorphisms

Although low-penetrance susceptibility genes may only result in a low to moderate increase in breast cancer risk, their presence along with endogenous and exogenous exposures, may have a greater role to play in breast carcinogenesis compared to that of high-penetrance genes such as BRCA1 and BRCA2.

Enzymes involved in metabolic pathways that either activate or inactivate carcinogens are of relevance to breast cancer risk (Okobia & Bunker, 2003). The cytochrome P450 (CYP) family controls the metabolism of most drugs as well as all carcinogens. Polymorphisms in CYP450 genes can affect breast cancer risk. CYP2D6, when mutated, has also been shown to play a role in breast cancer susceptibility (Pontin *et al.*, 1990; Topic *et al.*, 2000).

Mutations in members of the Glutathione S-transferase (GST) superfamily can lead to the absence of isoenzymes which can lead to the impairment of the body's responses to chemical challenges, and thus influence cancer susceptibility. Some studies have found a correlation between GST gene mutations and a moderate increase in breast cancer risk (Gudmundsdottir *et al.*, 2001). In addition to this, mutations in the MTHFR (5-10-methylenetetrahydrofolate reductase) enzyme (responsible for DNA synthesis and maintenance), and the XRCC1 gene (involved in DNA repair) have been associated with an increase in breast cancer risk (Campbell *et al.*, 2002; Ergul *et al.*, 2003; Smith *et al.*, 2003).

Alcohol dehydrogenase (ADH), a rate-limiting enzyme in alcohol oxidation may affect breast cancer risk, since alcohol is a well known risk factor. One study found that premenopausal women homozygous for the ADH1C*1 allele were at a 1.8 times higher risk for breast cancer compared to women with the other two genotypes (Freudenheim *et al.*, 1999).

1.5 Genetics of sporadic breast cancer

Approximately, 5% of all breast cancers occur due to inherited germline mutations. The remaining 95% are due to acquired genetic changes. The genes which undergo sporadic mutation are mostly the tumour suppressor genes and oncogenes but also of importance are changes in genes that are involved in cell-cycle inhibition, cell-cell adhesion, angiogenesis, DNA repair and apoptosis (for a review see (Kenemans, 2004).

While BRCA1 mutations account for a high proportion of hereditary breast cancers, BRCA1 mutations are rare in sporadic breast cancers. However, in sporadic cases BRCA1 is underexpressed in about 30% of cases (Yoshikawa *et al.*, 1999). Functional impairment is common, often the result of hypermethylation of the BRCA1 promoter (Dobrovic & Simpfendorfer, 1997; Esteller *et al.*, 2000). Loss of heterozygosity (LOH) of the region on chromosome 17, which includes BRCA1, has been found in many sporadic breast cancers (Beckmann *et al.*, 1996; Hanby *et al.*, 2000; Johnson *et al.*, 2002). Likewise, mutations in the BRCA2 gene are rare in sporadic breast cancer (Lancaster *et al.*, 1996), but LOH in chromosome 13q12 region has been found in 20-54% of sporadic cases (Cleton-Jansen *et al.*, 1995; Hanby *et al.*, 2000; Johnson *et al.*, 2002).

Mutations in p53 are found in approximately 50% of all human cancers (Malkin, 1994) and around 90% of carriers of the mutation develop cancer by the age of 70. P53 mutations appear in 20-60% of sporadic breast cancers (Deng *et al.*, 1994; Osborne *et al.*, 1991). Aberrations of p53 have been associated with a poorer prognosis in breast cancer patients (Thor *et al.*, 1992). Like the BRCA genes, p53 shows a high frequency of LOH in breast cancer (Hanby *et al.*, 2000; Johnson *et al.*, 2002).

The human epidermal growth factor receptor 2 gene (HER-2, ErbB2/neu) is localised to chromosome 17q and encodes a transmembrane tyrosine kinase receptor protein that regulates cell proliferation, differentiation, and survival. Amplification and overexpression of HER-2 has been reported in up to 25% of invasive breast cancers (Slamon *et al.*, 1987) and DCIS, where it acts as an oncogene to promote progression of the cancer, and is associated with high grade cancers and those with positive lymph node status (Burstein, 2005).

The c-myc oncogene has been localised to chromosome 8q24 and encodes a nuclear phosphoprotein that acts as a transcriptional regulator involved in cellular proliferation,

differentiation, and apoptosis (Dang, 1999; Liao & Dickson, 2000). C-myc is amplified and overexpressed in 15-25% of breast tumours (Deming *et al.*, 2000) and, has been associated with worse prognosis (Mizukami *et al.*, 1995).

Cyclin D1 belongs to the family of cyclin proteins which function as the regulatory subunits of cyclin/cyclin dependent kinases (Cdks) that regulate entry into and progression through the cell cycle. Cyclin D1 overexpression has been reported in between 40 and 90% of cases of invasive breast cancer, while gene amplification is seen in about 5–20% of tumours (Gillett *et al.*, 1994; Weinstat-Saslow *et al.*, 1995; Zukerberg *et al.*, 1995). Evidence to support that overexpression of cyclin D1 is a good prognostic factor varies greatly. Some studies have shown that overexpression of cyclin D in invasive breast cancer is associated with better disease-free intervals and overall survival, particularly for ER-positive patients (Bilalovic *et al.*, 2005; Gillett *et al.*, 1996), while others has found that gene amplification relates to a poorer prognosis in ER positive patients (Michalides *et al.*, 1996; Seshadri *et al.*, 1996).

The gene encoding cyclin E is located on chromosome 19q12. Cyclin E is rarely amplified in breast cancer, however the protein product is overexpressed in ~40% of breast cancers (Keyomarsi *et al.*, 1994; Loden *et al.*, 2002). In their study, Keyomarsi *et al.* found the expression of cyclin E to correlate with decreased disease-specific survival. Cyclin D1 appears to be overexpressed predominantly in ER+ cancer, whereas Cyclin E is predominantly overexpression is confined to ER- breast cancers (Buckley *et al.*, 1993; Loden *et al.*, 2002).

Somatic E-cadherin mutations are normally found in infiltrating lobular carcinomas or *in situ* lobular carcinoma types than in other histological types (Mastracci *et al.*, 2005). A number of studies have found a correlation between loss of E-cadherin expression and adverse outcome in breast cancer (Charpin *et al.*, 1998; Parker *et al.*, 2001; Yoshida *et al.*, 2001), while others have found E-cadherin expression to be a marker for disease progression (Gillett *et al.*, 2001; Jones *et al.*, 1996; Oka *et al.*, 1993).

1.6 Tumour characteristics

1.6.1 Pathology

In situ cancer confine themselves to the ducts or lobules and do not spread to the surrounding tissues in the breast or other parts of the body. These cancers include ductal carcinoma *in situ* (DCIS), and lobular carcinoma *in situ* (LCIS). DCIS is thought to account for only a very small proportion of breast cancers (5%). It is characterised by proliferation of malignant epithelial cells within the mammary duct, with no evidence of invasion into the surrounding stroma. LCIS lesions can be localised or extensive, and can also be bilateral in some cases. Both DCIS and LCIS can only be identified histologically and are considered to be risk lesions, although both may also be precursor lesions (WHO, 2003).

Invasive (or infiltrating) cancers have the potential to metastasise and can therefore be life threatening. Invasive ductal and lobular breast carcinomas are the most common breast malignancies, representing ~80% and ~15% of invasive breast carcinomas respectively (Joensuu & Toikkanen, 1995). Other less common types include mucoid or colloid (2.4%, tubular (1.2%), adenoid cystic (0.4%), cribriform (0.3%), and carcinosarcoma (0.1%).

1.6.2 Prognosis

Prognostic factors that are used clinically for patients with breast cacner are those that forecast the most likely clinical outcome. Factors used include tumour type, (e.g. infiltrating ductal carcinoma, infiltrating lobular carcinoma), tumour grade, tumour size, lymph node status, and presence of distant metastasis. The TNM staging system developed by the American Joint Committee on Cancer Staging (Singletary, 2002) is an important tool because its assesses the extent to which the tumour has spread, and is therefore a powerful predictor of survival. The system utilises the size of a primary tumour (T), whether axillary lymph nodes do or don't contain metastases (N) and whether the cancer has spread to a different part of the body (M). The system uses numbers to describe the cancer. 'T' can be 1-4, with '1' being a small tumour (tumour 20 mm or less) and '4' a large one (tumour greater than 100 mm in diameter), 'N' can be 0-3 with '0' meaning no positive lymph nodes and '3' many positive nodes, and 'M' is either 0 or 1 with '0' meaning no spread and '1' meaning that there is spread.

Table 1.1 The TNM system to stage cancer progression

Stage	Description
T_1	Tumour 20mm or less.
T_2	Tumour 20-50mm or less than 20mm but with tethering.
T_3	Tumour greater than 50mm but less than 100mm.
T_4	A tumour of any size with ulceration or infiltration wide of it, or chest wall fixation, or greater than 100mm in diameter.
N_0	Node negative
N_1	Axillary nodes mobile
N_2	Axillary nodes fixed
M_0	No distant metastases
M_1	Distant metastases

Grading refers to the histological differentiation of the tumour. Tumours are graded 1-3 depending on how differentiated the tumour cells appear. A 'low' grade cancer is one where the cancer cells have a well-differentiated appearance with low mitotic activity, whereas 'high' grade cancer has cells that have poor differentiation (Elston & Ellis, 1991).
The Nottingham Prognostic Index (NPI) incorporates tumour size, stage, and grade and today it is the most widely used tool to predict survival and the clinical course of the disease (Galea *et al.*, 1992).

The formula is:

NPI = (0.2 x tumour diameter in cms) + lymph node stage + tumour grade

The scores for tumours fall into 4 categories:

%: 5 year survival rate

Scores ≤ 2.4	95%
Scores between 2.4 and 3.4	85%
Scores between 3.4 and 5.4	70%
Scores between 4.4 and 5.4	50%
Scores >5.4	20%

1.6.3 Predictive Markers

1.6.3.1 Hormone Receptors

Immunohistochemistry tests for the oestrogen receptor (ER) and progesterone receptor (PgR) receptor are used in the clinical setting, since it provides an indication of the responsiveness to hormonal intervention, such as Tamoxifen. At present, the oestrogen receptor (ER) is probably the most powerful individual predictive factor examined in breast cancers. Almost 66% of women aged <50 years will have ER positive breast cancer, whereas approximately 80% of tumours in women >50 years old are ER positive (Anderson *et al.*, 2002). Patients with ER-positive breast cancers respond well to endocrine therapy, although response rates are lower in those with metastastic disease (Osborne, 1998). Patients with ER negative cancers have shorter disease-free intervals, earlier recurrence rates, and shorter survival when compared with ER positive cancers (Crowe *et al.*, 1991; Maynard *et al.*, 1978).
PgR is an oestrogen-regulated gene, and is thought to be a sign of a functioning ER pathway. Patients with PgR negative cancers also have shorter disease-free interval, and shorter survival compared to those with PgR positive cancers (Chevallier *et al.*, 1988; Fisher *et al.*, 1988).

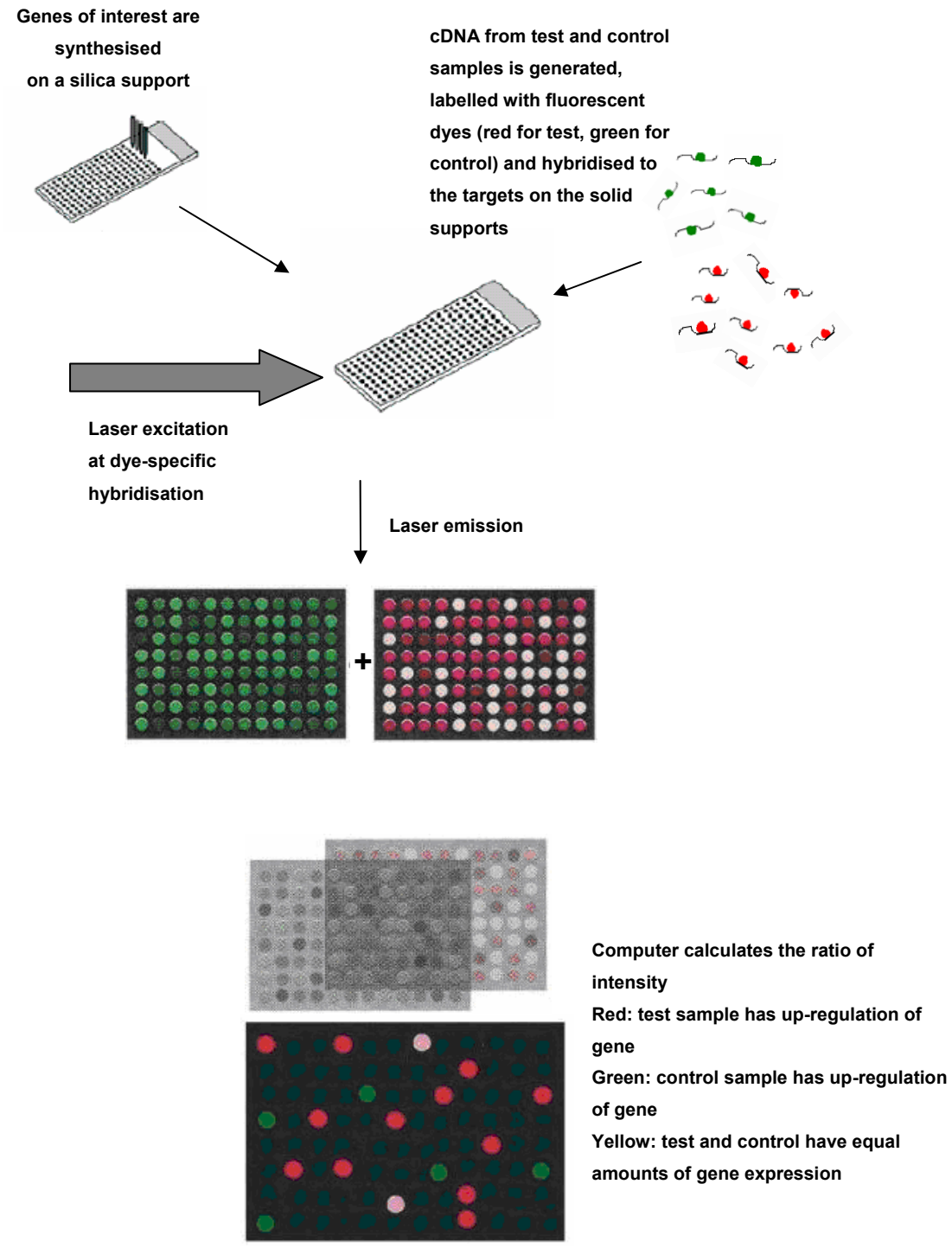
1.6.3.2 Oncogenes

In breast cancer HER-2 is related to earlier recurrence and mortality rates, and thus a poorer prognosis (Yamauchi *et al.*, 2001). HER-2 status predicts response to various forms of systemic chemotherapy and treatment with trastuzumab (more commonly known under the trade name Herceptin™). Recently, several clinical trials have revealed that trastuzumab in the adjuvant setting significantly reduces the risk of recurrence and mortality in patients with early-stage breast cancer (Joensuu *et al.*, 2006; Romond *et al.*, 2005; Smith *et al.*, 2007). In the UK, trastuzumab has been recommended for early-stage and metastatic breast cancer, but only for HER-2 positive cancers. At present, HER-2 status is determined by immunohistochemistry at the protein level and fluorescence in situ hybridization (FISH) at the genetic level.

1.7 Microarray technology and breast cancer

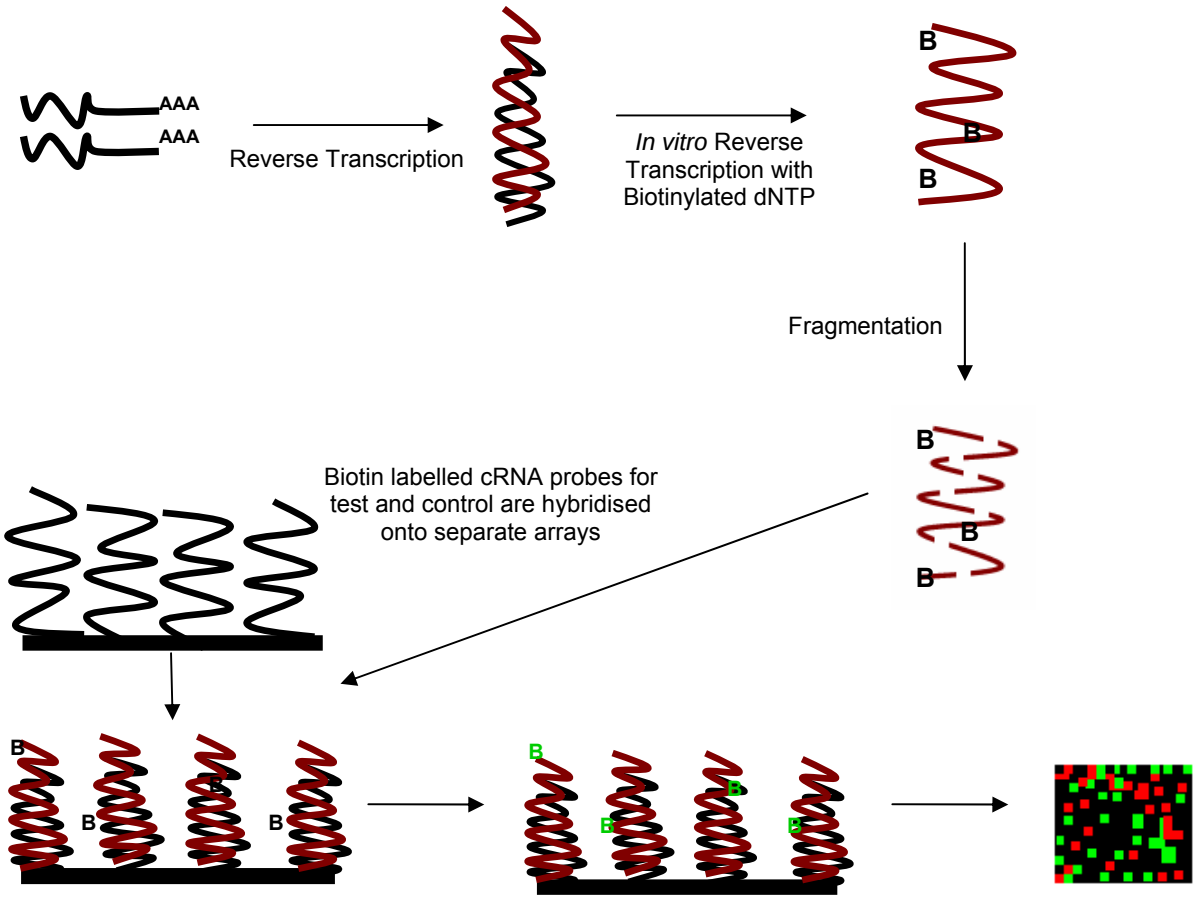
With the completion of the Human Genome Project, around 30-40,000 genes have been identified (IHGSC, 2004). This new information along with the development of DNA microarray technology is facilitating the study of gene expression and the roles played by these genes in many diseases including cancers. Micro array technology is a relatively new method that allows the simultaneous measurement of expression levels of thousands of genes. A microarray is a systematic arrangement of thousands of DNA probes (representing individual genes) attached onto a solid support. It is then probed with labelled DNA or RNA samples and the intensity of hybridisation is quantitated using computer software (for overview, see Figure 1.2) as a measure of DNA copy number and gene expression respectively.

Figure 1.2 (a) Schematic overview of the principle of cDNA microarray



Coloured microarray chips in this illustration were taken from the University of Newfoundland Department of Biology webpage: (http://www.mun.ca/biology/scarr/cDNA_microarray_assay_of_gene_expression.html)

(B) Overview of Affymetrix GeneChips™



Binding of a streptavidin-conjugated fluorescent marker allows the array to be read using a scanning confocal laser

One of the most commonly used microarrays is the GeneChip® array produced by Affymetrix Inc. GeneChip® arrays are made via *in situ* synthesis of oligonucleotide probes on a silica support using photolithographic techniques. The photolithographic masks act to block or transmit light onto specific locations on the chip. This chip is then flooded with a given nucleotide (A, C, G or T) and coupling occurs only to the illuminated areas. This step is repeated until the required number of oligonucleotides are synthesised.

Identifying novel and known genes that are differentially expressed in breast cancer has important implications in understanding the biology of breast tumourigenesis and developing new diagnostic and therapeutic agents. Since DNA microarray technology provides a way of measuring the expression of thousands of genes at any one time, it may revolutionise our approach to breast cancer diagnosis, prognosis and treatment.

One of the first studies that characterised the variation in gene expression between sporadic breast tumour samples was published by Perou *et al.* (1999). This landmark study was the first to establish that tumours could be phenotypically categorised into subtypes distinguished by differences in their expression profiles. In the first study, 40 breast cancers, and 20 matched pairs of cancers before and after doxorubicin treatment were examined. An 'intrinsic genes set' of 476 cDNAs were selected that were more variably expression between the 40 sporadic tumours than between the paired samples (Perou *et al.*, 1999). This intrinsic set was then used to cluster and segregate the tumours into four major subgroups: (1) a 'luminal cell-like' group expressing ER, (2) a 'basal cell-like' group expressing keratins 5 and 17, integrin β4, and laminin, but lacking ER expression, (3) a HER-2-positive subgroup, and (4)a 'normal' epithelial group (Perou *et al.*, 2000). Subsequent studies by this group have since extended the molecular profiling of breast cancers by applying their intrinsic gene set to cluster 78 cancers, 3 fibroadenomas, and 4 normal breast tissue samples (Sorlie *et al.*, 2001). The same subgroups were found as before (Perou *et al.*, 1999; Perou *et al.*, 2000), except the luminal, ER-positive group was sub divided into two groups, luminal A, and luminal B. To explore whether the five different tumour subgroups identified may represent clinical distinct and relevant groups of patients, univariate survival analyses with respect to overall survival and relapse-free survival was performed. A significant difference in overall survival belonging to the different subgroups was found. In particular, the basal-like, and HER-2-positive, subgroups were associated with the shortest survival time.

Since then, many other groups have investigated the prognostic value of different gene signatures (Ahr *et al.*, 2002; Bertucci *et al.*, 2000; Sotiriou *et al.*, 2003; van 't Veer *et al.*,

2002; Wang *et al.*, 2005); (Bertucci *et al.*, 2002; Martin *et al.*, 2001; West *et al.*, 2001). One of the largest and most important of those studies was published by van't Veer *et al.* (2002). This group wanted to identify a poor prognosis Gene Expression Profile (GEP) for node negative breast cancers. They undertook cDNA microarray analysis of 117 patients (<55 years) and related expression profiles to short disease free interval. The resulting poor prognosis GEP included 70 genes (see Table 1.2), many of which were involved with cell cycle control, invasion, metastasis and angiogenesis. Evaluation of this prognostic profile was conducted in a follow-up study, applied to 234 primary breast carcinomas. Among the 234 patients, 180 had poor prognosis GEP and 115 had a good-prognosis GEP (van de Vijver *et al.*, 2002). It outperformed current clinical parameters in predicting disease outcome and confirmed the predictive power of the profile.

Other investigators have used gene expression profiling to: identify novel target genes in breast cancer (Bertucci *et al.*, 2000; Jiang *et al.*, 2002); predict response to therapies (Sotiriou *et al.*, 2002); compare ductal carcinoma *in situ* (DCIS) (Adeyinka *et al.*, 2002; Seth *et al.*, 2003)and medullary carcinoma (Vincent-Salomon *et al.*, 2007) to standard invasive carcinomas; compare the differences between lobular and ductal carcinomas (Korkola *et al.*, 2003); predict metastasis (Smid *et al.*, 2006; Thomassen *et al.*, 2007); identify genes associated with metastasis (Schwirzke *et al.*, 2001); correlate genomic alterations such as LOH and DNA copy number with gene expression profiles (Pollack *et al.*, 2002; Wang *et al.*, 2004); identify genes associated with HER-2 (Mackay *et al.*, 2003) and hormone receptor status (Nagai *et al.*, 2004); identify genes associated with BRCA1/2 tumours; and separate familial non-BRCA breast cancers into subgroups based on their gene expression profiles (Hedenfalk *et al.*, 2003). These are summarised in Table 1.3

Table 1.2 'Poor prognosis' signature of 70 genes taken from (van 't Veer *et al.*, 2002)

Gene Name	Gene Name
aldehyde dehydrogenase 4	peroxisomal D3,D2-enoyl-CoA isomerase
fibroblast growth factor 18	gene for serine/threonine protein kinase
ESTs	Homo sapiens hepatocellular carcinoma-associated antigen 64 (HCA64) mRNA
Bcl-2 binding component 3	high affinity immunoglobulin epsilon receptor beta subunit
KIAA1442 protein	adaptor-related protein complex 2, beta 1 subunit
CEGP1 protein	hypothetical protein FLJ11354
hypothetical protein FLJ10474	peroxisomal biogenesis factor 12
WNT1 inducible signaling pathway protein 1	GCN1 (general control of amino-acid synthesis 1, yeast)-like 1
glutathione S-transferase M3 (brain)	quinoid dihydropteridine reductase
ESTs, Weakly similar to T17248 hypothetical protein DKFZp586G1122.1	KIAA1181 protein
hypothetical protein MGC2827	Homo sapiens mRNA full length insert cDNA clone EUROIMAGE 44260
transforming growth factor, beta 3	acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain
methylmalonate-semialdehyde dehydrogenase	Homo sapiens mRNA; cDNA DKFZp564L0678
hypothetical protein FLJ12150	protein disulfide isomerase related protein
cold inducible RNA-binding protein	keratin 18
matrilin 3	myosin regulatory light chain interacting protein
phosphatidylinositol (4,5) bisphosphate 5-phosphatase, A	ESTs, Weakly similar to Homolog of rat Zymogen granule membrane protein
Homo sapiens cDNA: FLJ23228 fis, clone CAE06654	RAB27B, member RAS oncogene family

ESTs, Moderately similar to hypothetical protein [H.sapiens]	KIAA0882 protein
ESTs, Weakly similar to DWHUT L-serine dehydratase	DKFZP586A011 protein
cholinephosphotransferase 1	paired basic amino acid cleaving system 4
ESTs, Weakly similar to unnamed protein product	SEC14 (S. cerevisiae)-like 2
Homo sapiens mRNA; cDNA DKFZp434E2321 (from clone DKFZp434E2321)	hypothetical protein DKFZp761L0424
fructose-1,6-bisphosphatase 1	retinol-binding protein 3, interstitial
TBX3-iso protein	kinesin family member 3B
KIAA1324 protein	DKFZP586F1018 protein
ribosomal protein S4, X-linked	DKFZP434I114 protein
BTG family, member 2	retinoic acid induced 2

15 of the 70 genes were ESTs (Expressed sequence tags). No information exists for these genes

Table 1.3 Summary of cDNA microarray studies and their findings

Findings	Reference
Tumour Sub grouping	
Segregated tumours into 4 major subgroup: (1) a 'luminal cell-like' expressing ER, (2) a 'basal cell-like' expressing keratins 5 and 17, integrinβ4, and laminin, but no ER, (3) a HER-2-positive subgroup, and (4) a 'normal' epithelial group.	(Perou *et al.*, 1999; Perou *et al.*, 2000)
Successfully clustered 78 cancers, 3 fibroadenomas, and 4 normal samples using their intrinsic gene set. Also found that the 'luminal' ER-positive subgroup was itself divided into a luminal A and a luminal B.	(Sorlie *et al.*, 2001)
Disease Outcome/survival	
Found that the 'basal' and 'HER-2' type breast cancers had a poor prognosis	(Sorlie *et al.*, 2001)
Found gene expression signatures that correlated survival	(Ahr *et al.*, 2002), (Bertucci *et al.*, 2000; Bertucci *et al.*, 2002), (Sotiriou *et al.*, 2003), (van 't Veer *et al.*, 2002), (Wang *et al.*, 2005), (West *et al.*, 2001), (Martin *et al.*, 2001)
Novel Targets	
Identified new ways to group tumours according to outcome and new potential targets of carcinogenesis	(Bertucci *et al.*, 2000)
Identified novel candidate genes in sporadic breast cancer	(Jiang *et al.*, 2002)

Response to therapies	
Identified candidate gene expression profiles that might distinguish tumors with complete response to chemotherapy from tumors that do not respond	(Sotiriou *et al.*, 2002)
Analysis of tumour types	
DCIS	(Adeyinka *et al.*, 2002)
Medullary breast carcinoma - found it to be part of the 'basal' like group	(Vincent-Salomon *et al.*, 2007)
Lobular v ductal carcinomas	
Found specific changes in gene expression that distinguish lobular from ductal breast carcinomas	(Korkola *et al.*, 2003)
Other analyses	
Gene expression profiles of ER-pos/neg and PgR-pos/neg breast cancers	(Nagai *et al.*, 2004)
Discovered distinct expression profiles in breast tumours from BRCA1 and BRCA2 mutation carriers. Also discovered novel classes among non-BRCA tumours, and differentiate them from BRCA1 and BRCA2 tumouurs	(Hedenfalk *et al.*, 2003)
Used gene expression profiling to predict metastasis, and metastasis-associated genes.	(Smid *et al.*, 2006; Thomassen *et al.*, 2007), (Schwirzke *et al.*, 2001)
Using gene expression profiling this group found that at least 12% of all the variation in gene expression among the breast tumours is directly attributable to underlying variation in gene copy number.	(Pollack *et al.*, 2002)
Identified genes associated with HER-2	(Mackay *et al.*, 2003)

Comparative genomic hybridisation (CGH) has been used to study genomic imbalances in breast cancer in younger women. After using CGH to look at 88 cancers in women ≤ 35 years, Weber-Mangal *et al.* (2003) found genomic gains at chromosome arms 1q (64.8%), 8q (61.4%), 17q (50%), 20q (33%), 3q (20.5%), 1p (17%), 5p (17%), an 15q (17%). There were genomic losses at chromosomes 8p (19.3%), 11q (11.4%), 16q (11.4%), 17p (11.4%) and 18q (10.2%). When the data were compared to data from breast cancers from older women (see Table 1.4 for a summary), they found that genomic losses were more common than gains in younger women. Losses on 8p22-p23 were more prevalent in patients with positive lymph node metastasis, and Grade III tumours were associated with gains on the long arm of chromosome 8.

Table 1.4 Summary of CGH studies in breast cancer

Gains	Losses	Tissue studied	References
1q , 8q, 17q, 20q, 3q, 1p , 5p and 15q	8p , 11q, 16q, 17p and 18q	Tumours in women ≤35 years	(Weber-Mangal *et al.*, 2003)
1q, 8q24, 11q13, 17q21-q23, and 20q13	N/A	Analysis of breast tumour progression	(Yao *et al.*, 2006)
1q , 8q, 16p – Grade I, and 1q, 8q, 17q, 10q, 16p – Grade II	16q – Grade I	Primary tumours, correlated by grade	(Roylance *et al.*, 1999)
8q-,1q31, 20q12, 8q13, 3q26, 17q21, 5p14, 6p22, and 22p	13q21, 8p12, 4p13-p14, 6q15-q22, and 18q11	Primary tumours and their cell lines	(Larramendy *et al.*, 2000)
1q, 17q, 19q, 20p and 20q	13q, 14q, 17p, 16q and 22q	DCIS and IDC	(James *et al.*, 1997)
1q, 7q, 3q,1p, 2q, 5p, 8q, and 13q	17p	DNA ploidy breast tumours	(Pinto *et al.*, 2006)
1p, 1q, 6p, 7p, 8q, 9q, 11q, 12q, 17p, 17q, 20q, and 22q	6q, 9p, 11q, and 17p	Primary tumours	(Nessling *et al.*, 2005)
1q, 8q, 16p, 5p12-14, 19q, 11q13-14, 17q12, 17q22-24, 19p, and 20q13	8p, 16q, 13q, 17p, 9p, Xq, 6q, 11q, and 18q	Primary tumours	(Tirkkonen *et al.*, 1998)
1p, 4q, 5q, 6q and 13q	N/A	Tumour histological subtypes	(Loveday *et al.*, 2000)
17q , 1q, 8q, 20q, 6p	13q, 11q, 5q, 6q, 9p, 18q, 8p, and 16q	Prognostic relevance	(Seute *et al.*, 2001)

N/A: data not shown in publication

1.8 Breast cancer in younger women

The incidence of breast cancer is very low before age 35 with only 2.5% occurring in women less than 35 years, and 0.6% in women less than 30 years (Ries *et al.* 2004). The incidence increases up to 100-fold by age 45 (Hulka & Moorman, 2001). Breast cancers in young women are normally infiltrating ductal carcinomas with invasive lobular types less common (Kollias *et al.*, 1997). The mortality rates among women ≤ 35 years are much higher compared with women >35 years, with the 10-year survival rates at 35% and 47% respectively (Feldman & Welch, 1998; Winchester *et al.*, 1996). The 30-year survival rate falls to 19% in women under 30 years (Feldman & Welch, 1998).

1.8.1 Comparison of biomarkers among younger and older women

This poorer prognosis in women ≤ 35 years with breast cancer is thought to be due to differences in tumour biology. Several studies have carried out direct comparisons of the pathology and biomarkers in cancers from younger women with cancers from older women, and have found that those from the younger women tend to have more biologically aggressive features. Breast cancers in younger women have high incidence of BRCA1/2 mutations (15-30% of cases) (Tilanus-Linthorst *et al.*, 2007), but many are also sporadic. Younger women present with much higher grade tumours (Colleoni *et al.*, 2002; El Saghir *et al.*, 2006; Fernandopulle *et al.*, 2006; Figueiredo *et al.*, 2006; Gonzalez-Angulo *et al.*, 2005; Kollias *et al.*, 1997; Walker *et al.*, 1996) (For a summary please refer to Table 1.5). There is considerable evidence that tumours from women ≤ 35 years tend to be negative for the oestrogen receptors (ER) and progesterone receptors (PgR) (Ahn *et al.*, 2007; Colleoni *et al.*, 2002; Figueiredo *et al.*, 2006; Gonzalez-Angulo *et al.*, 2005; Hartley *et al.*, 2006; Walker *et al.*, 1996). In contrast, a few studies have found higher proportions of ER positive and PgR positive tumours in younger women (El Saghir *et al.*, 2006; Fernandopulle *et al.*, 2006). Tumours from women ≤ 35 years were also found to have high levels of Ki-67 (a proliferation marker) (Colleoni *et al.*, 2002; Hartley *et al.*, 2006), positive nodal status (Ahn *et al.*, 2007; Fernandopulle *et al.*, 2006), and lymphatic or vascular invasion (Colleoni *et al.*, 2002; Kollias *et al.*, 1997) (Fernandopulle *et al.*, 2006; Figueiredo *et al.*, 2006; Jmor *et al.*, 2002). HER-2 is overexpressed in 25-30% of all invasive breast cancers (Slamon, 1990). Tumours in patients ≤ 35 years and those >35 years appear to have similar levels of HER-2

expression (Bertheau *et al.*, 1999; Choi *et al.*, 2005; Colleoni *et al.*, 2002), although two studies have found high HER-2 expression in tumours from women ≤ 35 years (Hartley *et al.*, 2006; Maru *et al.*, 2005). Maru *et al.* also found a correlation between HER-2 expression and the incidence of lymph node metastasis.

With regard to p53 status, a number of groups have found an abnormal accumulation of the protein in tumours from women ≤ 35 years (Albain *et al.*, 1994; Barnes *et al.*, 1993; Walker *et al.*, 1996). (Bertheau *et al.*, 1998) found no correlation between p53 expression and age, but did find an association between p53 and shorter overall survival in patients ≤ 35 years.

The theory that age itself is a prognostic indicator for breast cancer has been examined. A large study found a higher frequency of large tumours, a significantly higher incidence of grade III tumours and microscopic lymph node involvement, and lack of ER and PgR in tumours from women ≤ 35 years (Bonnier *et al.*, 1995). Multivariate analysis of overall survival showed that age ≤ 35 years was an independent risk factor. These findings were supported by other studies (Adami *et al.*, 1986; El Saghir *et al.*, 2006). In contrast, a more recent study found that the actual adverse tumour characteristics found in tumours from women ≤ 35 years, was the poor prognostic indicator and not age as previously reported (Figueiredo *et al.*, 2006).

Younger women diagnosed with ductal carcinoma *in situ* (DCIS) appear to have a different natural history and biology, including a higher local relapse, compared to older women. A study looking into this disparity found that lesions in women < 42 years had a higher HER-2 expression compared to women >60 years (Rodrigues *et al.*, 2003). In both age groups, HER-2 expression was correlated with high nuclear grade, and negative hormone receptors.

Very few studies have looked at breast cancers in pregnant women. One study did however look at the pathology and expression of standard predictive biomarkers in 39 pregnant women from age 24 to 44 (Middleton *et al.*, 2003). The results found cancers in this category to be more advanced at initial diagnosis, and have higher grades, lymphovascular invasion, high Ki-67 staining, and largely lack of hormone receptors. A different study found that women ≤ 35 years with three or more childbirths were more likely to have a more advanced tumour at presentation and poorer survival rates compared to nulliparous women (Largent *et al.*, 2005). Those who lactated tended to have ER and PgR receptor negative tumours.

Table 1.5 Biological marker studies in breast cancer in young women

Author	Age	N	ER-negative	PgR-negative	Ki-67	Grade III	HER-2-positive
(Walker *et al.*, 1996)	25-29	18	66%	67%	75%	67%	22%
	30-34	30	43%	63%	67%	70%	20%
	35-49	40	30%	40%	40%	44%	22.5%
	40-44	75	25%	56%	50%	58%	17%
	50-67	70	30%	51.5%	40%	37%	17%
(Kollias *et al.*, 1997)	< 35	111				76%	
	35-50	941	N/A	N/A	N/A	47%	N/A
	51-70	1623				41%	
(Bertheau *et al.*, 1999)	<35	50	N/A	N/A	N/A	N/A	26%
	36-50	62					34%
(Colleoni *et al.*, 2002)	<35	185	39%	N/A	62%	62%	40%
	35-50	1242	21%		53%	37%	37%
(Jmor *et al.*, 2002)	<35	133	80%	N/A	N/A	73%	N/A
(Maru *et al.*, 2005)	23-30	44	55%	64%	N/A	68%	44%
(Gonzalez-Angulo *et al.*, 2005)	≤35		48%	52%	N/A	69%	34%
(Choi *et al.*, 2005)	25-45	103	58%	51%	39%	N/A	28%
(Fernandopulle *et al.*, 2006)	≤35	112	39%	48%	N/A	59%	29%
(El Saghir *et al.*, 2006)	<35	107	ER and/or PgR 29%		N/A	49%	N/A
	35-50	526	63%			42%	

	51-70	687		22%		35%	
(Figueiredo *et al.*, 2006)	≤35	105	40%	36%	N/A	68%	N/A
	>35	862	24%	28%		36%	
(Hartley *et al.*, 2006)	≤40	78	34%	50%	62%	N/A	44%
	>40	228	22%	35%	29%		23%
(Ahn *et al.*, 2007)	<35	1444	30%	32%	N/A	N/A	N/A
	35-50	8441	27%	27%			

1.8.2 Racial/Ethnic variation in biological features in younger women

The age specific breast cancer incidence rate for African-American women ≤ 35 years is more than double the rate for white women of similar age, and the mortality rate is more than three times higher (Elledge *et al.*, 1994; Elmore *et al.*, 1998). In one study a US based group sought to examine this racial/ethnic variation. To do this, they studied the clinical presentation and survival among a large number of African-American, Hispanic, and white women ≤ 35 years with breast cancer. The study found that in the Hispanic and African-American populations the tumours were more aggressive. Compared with white women, African-American and Hispanic women presented with tumours that were poorly differentiated, aneuploid, and had higher S-phase fractions, (Shavers *et al.*, 2003).

A different study looked at the biological differences between white women ≤ 45 years in the USA, and age matched native Korean women (Choi *et al.*, 2003). After examining ER, PgR, p53, HER-2, and cyclin D, they found a significant difference in the expression of HER-2 between the ethnic groups. HER-2 was positive in 47.5% of tumours from Korean women, compared to only 15.8% in tumours from white women.

1.8.3 BRAC1 and BRCA2

Mutations in the BRCA1 and BRCA2 genes are more common in younger women with breast cancer (Bonadona *et al.*, 2005). A study looking comparing BRCA-related and sporadic young breast cancers, found higher local recurrences as well as contra-lateral breast in the BRCA-related cases (Verhoog *et al.*, 1998).

Studies within my research group have found loss of heterozygosity (LOH) of BRCA1, BRCA2, and p53 to be higher in tumours from patients ≤ 35 years compared with those from patients >55 years (Johnson *et al.*, 2002).

1.8.4 Other biological features

A study examining the frequency of basal breast cancers (cancers lacking hormone receptors and HER-2 expression) in African-American and non-African-American found that the triple negative basal breast cancers were noticeably more common among African-Americans, particularly pre-menopausal African-American women (Carey *et al.*, 2006). A similar study also found the basal breast cancer subgroup to be more prevalent among younger African-

American women, which may also explain the poorer prognosis associated with this ethnic age-group (Ihemelandu *et al.*, 2007).

Another investigation that was prompted by the microarray data attempted to measure the expression of 16 breast cancer-related and 5 reference genes in order to generate a Recurrence Score. This recurrence score was clinically validated to quantify the risk of distant recurrence. Among a cohort of 447 patients, the percentage of women with a high RS score was higher for women ≤ 40 years, than for those aged 40-50, 50-60, and >60 years. In a multivariate Cox model, the RS proved to be a significant predictor independent of age and tumour size (Paik *et al.*, 2004).

1.9 Background to project

Interest in breast cancer in younger women was started by a study by (Walker *et al.*, 1996) where a group of 163 invasive breast carcinomas from women ages 44 years and less were compared to symptomatic tumours from women ages 50-67 years. Ten per cent of women in this study aged 35 years and less were recorded to have a member of the family with breast cancer, compared to 18% of women aged between 35 and 44 years who often had more than one relative affected. In this lower age group, 70% of the cases were grade III with no specialised carcinomas, and had a low incidence of hormone receptor positivity. In addition, 67% of cases less than 30 years were found to be positive for p53 immunohistochemistry. This observation was significantly different to carcinomas from women ages 40-44 years (40%) and 50-67 years (37%). Subsequently, the research group, seeking to gain a better understanding of the molecular alterations in breast cancers in women ≤35 years, examined LOH at three chromosomal intervals containing BRCA1, BRCA2 and p53. In doing so, a high incidence of LOH was observed at BRCA1 and BRCA2 (Johnson *et al.*, 2002). Following from this, a cDNA microarray experiment was carried out to offer a global overview of gene expression changes in breast cancers in younger women. An Affymetrix Human Genome UI33A GeneChip® microarray was used to compare cDNA from two tumour samples from women ≤35 years to normal breast tissue from reduction mammoplasties and the non-tumourigenic HBL-100 breast cancer cell line. The results for the two tumours were combined, and compared to the HBL-100 cell line, and the normal breast sample. Sixty-nine genes were found to be up regulated and 372 were down regulated in the two tumour samples by significance analysis of microarrays (SAM).

1.10 Hypothesis and aims

The hypothesis to be tested is that breast cancers in women aged ≤35 years are biologically different to those in women >35 years and this can be investigated through study of candidate genes expression. The cDNA microarray study sought to identify novel gene targets in breast cancers in women aged ≤35.

The specific aims of this project were:

1. To select a number of genes, found to be ranked highly by SAM analysis, and develop real-time quantitative PCR to investigate their mRNA expression in breast cell lines, normal breast tissue, as well in a larger cohort of breast tumour samples (≤35 v >35) years, and to determine whether these genes are of significance in breast cancers in women ≤35 years.

2. To assess protein expression from targets validated in (1) and to compare the data in relation to clinical pathological and biological markers.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Cells

2.1.1.1 Established Cell Lines

All cell lines were originally obtained from the American Type Culture collection (ATCC Rockville, MD, USA) and taken from batches held at the University of Leicester. HBL-100 was used as a non-tumourigenic breast cell line compared with five breast cancer cell lines MDA-MB-231, MCF-7, ZR-75-1, T47-D, MDA-MB-468, which differ in phenotype (see Table 2.1).

Table 2.1 Summary of cell lines and their characteristics

Cell line	Tissue origin	ER*	PgR*	P53†	Inv‡	Reference
HBL-100	Milk of a 27 yr old Caucasian ♀nursing mother	+	+	Wt	22.7%	(Gaffney, 1982)
MDA-MB-231	PE from 51 year old Caucasian ♀with adenocarcinoma	-	-	M	42.1%	(Cailleau *et al.*, 1978)
MCF-7	Malignant PE of a 69 ♀ yr old with IDC	+	+	Wt	0.9%	(Brooks *et al.*, 1973)
ZR-75-1	Ascitic fluid from 63 yr old ♀with IDC	+	+	Wt	No data	(Engel *et al.*, 1978)
T47-D	PE secondary to IDC in a 54 yr old ♀	+	+	M	2.2%	(Keydar *et al.*, 1979)
MDA-MB-468	PE from 51 yr old Black ♀with adenocarcinoma	-	-	M	16.2%	(Cailleau *et al.* 1978)

IDC, infiltrating ductal carcinoma. PE, pleural infusion.
*ER, oestrogen receptor. PgR, progesterone receptor. Receptor status data from above references and (Horwitz *et al.*, 1978).
† Wt, wild-type allele. M, mutant allele. p53 mutation data from (Nigro *et al.*, 1989).
‡ Inv, mean invasion index (MII) data from and described in (Gordon *et al.*, 2003).

2.1.1.2 Normal Cells

Normal breast epithelial cells were obtained from reduction mammoplasty specimens, from women aged 21 to 42 with a median age of 31.5 years old. All samples were anonymised and each patient gave informed patient consent.

2.1.2 Tumour Tissue

All specimens were obtained fresh within 30-60 minutes after surgery for breast cancer (either wide local excision or mastectomy), and sliced to ensure optimal fixation. For 24 cases, 10 X 5 X 3mm were frozen in liquid nitrogen and stored in the vapour phase of a liquid nitrogen fridge. For all cases 3mm thick tissue blocks were selected by a pathologist after 18-24 hours of fixation in 4% formaldehyde saline, processed through graded alcohols and xylene, followed by embedding in paraffin wax.

A total of 66 breast cancer cases were studies, 36 for gene expression analysis and 56 for immunohistochemistry, the limiting factors being availability of appropriate tissue. All pathological data was available from reports and checked by Prof. Walker, and NHS BSP Breast Screening Pathology Guidelines were followed. Data was available for oestrogen receptor (ER) and progesterone receptor (PgR). There was ethical approval for the use of anonymised tissues.

2.1.3 Cell Culture

2.1.3.1 Complete Growth Media

- Dulbecco's modified Eagle's medium (DMEM) – (DMEM- Sigma-Aldrich) without phenol red, 2mM L-glutamine (Sigma-Aldrich), 10% v/v foetal calf serum (Labtech®).
- RPMI-1640 medium – RPMI-1640 medium (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 10% v/v foetal calf serum (Labtech®).

2.1.3.2 Washing Solutions and Reagents

- Phosphate buffered saline pH 7.4 (PBS – Sigma-Aldrich)
- Trypsin EDTA (Invitrogen™ Life Technologies)

- Dimethyl sulphoxide (DMSO – Sigma-Aldrich)

2.1.3.3 Enzymes and Antibiotics

- Collagenase Type IV 433U/mg (Gibco®)
- Hyaluronidase Type IV 5mg/mL (Sigma®)
- Penicillin Streptomycin (Sigma ®)
- Fungizone Amphotericin B 250µg/mL (Gibco®)

2.1.4 RNA Preparation

2.1.4.1 Total RNA isolation

- TRI Reagent® (Sigma-Aldrich), for single-step total RNA isolation (Chomczynski and Sacchi, 1987)
- Chloroform (Fisher Scientific®)
- Ultra-pure glycogen (Invitrogen™ Life Technologies)
- Isopropanol and 70% ethanol (Fisher Scientific®)
- Xylene
- 99% EtOH
- 95%EtOH
- Proteinase K solution (10mg/mL)
- 0.01M Tris pH 8
- 0.1mM EDTA
- 2% Sodium dodecyl sulfate
- 10 × TURBO DNase buffer (Ambion ®)
- TURBO DNAse (Ambion ®)
- Inactivation reagent (Ambion ®)

2.1.5 RT-PCR

2.1.5.1 Reverse Transcription

All reagents from Promega®:

- Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT), 20 U/µL.
- Recombinant Rnasin® Ribonuclease Inhibitor, 40U/ µL.
- Random Primers, 0.5µg/ µL.
- DNTP Mix, 10mM – equal concentrations of dATP, dCTP, dGTP and dTTP.
- Reverse Transcription 10x Buffer 100 mM Tris-HCL (pH 9.0 at 25°C), 500mM KCl and 1% Triton® X-100.
- Magnesium Chloride ($MgCl_2$), 25 mM
- Nuclease-free water

2.1.5.2 Polymerase Chain Reaction

- 10 × Alex Jeffrey's buffer (in house) – 450 mM tris-HCl(pH 8.8 - Fisons®), 110mM $(NH_4)_2SO_4$ (Fisons®), 45 mM $MgCl_2$ (Fisons®), 2mM dNTPs (dATP, dCTP, dGTP and dTTP - Roche® Applied Science), 67mM β-Mercaptoethanol (βME – Boehringer Mannheim), 5nM EDTA pH 8.0 (Fisher Scientific), 1.1 µg/mL Bovine Serum Albumin (BSA – Advanced Protein Products Ltd.) made up in DEPC H_2O.
- Sterile molecular biology water (Sigma-Aldrich®).
- *Taq* DNA polymerase (Promega®), 5U/µL.
- Primers were designed as described in section 2.2.4 and supplied by Genoysys® Biotechnologies, Europe. Primers were used at a working concentration of 10 pmol/µL. A summary of primers is provided in Table 2.3.

2.1.5.3 Horizontal gel electrophoresis

- Seakem® LE agarose powder (Cambrex®).
- 1X Tris-Acetate-EDTA – 2M Tris, 1M glacial acetic acid, 0.5 EDTA pH 8.0 all from Fisher Scientific®.
- Gel loading buffer – xylene cyanol 0.2%, bromophenol blue 0.2%, glycerol (Gibco-BRL®).
- 100bp DNA ladder (Invitrogen® - 1 µg/µL in 10mM Tris-HCL (pH 7.5), 1 mM EDTA.
- Ethidium bromide (Sigma-Aldrich®) 10 mg/mL.

2.1.6 Real time quantitative RT-PCR

2.1.6.1 SYBR Green RTqPCR

All reagents from Invitrogen®:

- SYBR ® Green I, 60 U/mL Platinum® *Taq* DNA polymerase, 40 mM Tris-HCl (pH 8.4), 100 mM KCl, 6 mM MgCl2, 400 µM dGTP, 400 µM dATP, 400 µM dCTP, 400 µM dUTP, 40 U/mL UDG, and stabilizers.
- Internal Reference Dye 50 X
- Magnesium Chloride ($MgCl_2$), 25mM.

2.1.6.2 Taqman Probes

All reagents from Applied Biosystems®:

- TaqMan® Fast Universal PCR Master Mix
- 18S probe

2.1.7 Antibodies

2.1.7.1 Primary Antibodies

- NCOA3 (Abcam®). A mouse monoclonal antibody raised against a fusion protein corresponding to amino acids 605-1294 of human NCOA3. Suitable for detection of NCOA3 of human origin by western blotting, immunoprecipitation and immunohistochemistry.
- RARRES3 (Abnova®). A mouse polyclonal antibody raised against a partial recombinant RARRES3 protein. Suitable for detection of RARRES3 of human origin by western blotting.
- Beta-Actin (Sigma ®). A mouse monoclonal anti-beta-Actin antibody.

2.1.7.2 Secondary Antibodies

- Anti-mouse Ig, horseradish peroxidise linked with whole antibody (Amersham®).
- Rabbit anti-mouse biotinylated (RαmBt) (DAKO Ltd)

2.1.7.3 Tertiary Reagents

- Streptavidin-biotin complexes (Strept ABC) labelled with horseradish peroxidise (DAKO Ltd)

2.1.8 Western Blotting

2.1.8.1 Reagents

- Gold Lysis buffer – 1% v/v Triton X-100, 20mM Tris pH 8.0, 137 mM NaCl, 15% v/v glycerol, 5mM EDTA.
- 100 × Protease inhibitor cocktail (Sigma-Aldrich®).
- Enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences®).
- Protein Standard (Bio-rad Laboratories®) – bovine serum albumin.

- Protein Assay Dye Reagent (Bio-rad Laboratories®) – based on Bradford dye-binding procedure (Bradford, 1976), contains Coomassie Brilliant Blue G-250 dye.
- Precision Plus Dual Colour Protein Size Marker (Bio-rad Laboratories®).
- 10% w/v ammonium persulphate (APS) (Sigma-aldrich®)
- TEMED (Sigma-aldrich®).
- Phosphate buffered saline pH 7.4 (PBS – Sigma-Aldrich)
- 30% H_2O_2 (Sigma-Aldrich)

2.1.8.2 Buffers and Stock Solutions

- Transfer buffer – 0.25 M Tris, 1.92 M Glycine, 0.01% w/v SDS, at pH 8.3.
- Washing buffer – TBS plus 0.1% w/v TWEEN 20
- Loading buffer – 1mL 0.5 M Tris-HCL, pH 6.8, 0.8mL glycerol, 1.6mL 10%SDS, 0.4 mL 2-mercaptoethanol, 0.2mL 0.05% bromophenol blue, 4 mL distilled H_2O.
- Blocking Solution – Washing buffer with 5% Marvel milk powder.

2.1.8.3 Membranes

- Nitrocellulose membrane (Amersham Hybond™ ECL™)

2.1.9 Immunohistochemistry

1.1.1.1 Reagents

- 10 × DAKO antigen retrieval solution pH8.8 diluted 1:10 in distilled water (DAKO Ltd)
- 2% hydrogen peroxide (H_2O_2) in distilled water
- Normal rabbit Serum (NRS) diluted 1:5 in TBS (Invitrogen Life Technologies)
- 3'3-diaminobenzidine (DAB) (Sigma-Aldrich) – A 500 µL DAB aliquot was diluted in 9.5mL distilled water and 100 µL 3% H_2O_2 was added just before use
- DPX mountant (VWR International)

2.1.9.1 Buffers and Washing Solutions

- Xylene and graded (95% and 99%) industrial methylated spirits (IMS) (Genta Medical)
- 1 × TBS, pH 7.65
- 1 × TE , pH 9.00
- Mayer's Haematoxylin solution – 0.1% Haematoxylin, 0.02% sodium iodide, 5% ammonium/potassium alum, 5% chloral hydrate and 0.1% citric acid
- Vectabond solution

2.2 Methods

2.2.1 Cell Culture

All cell cultures were grown and sub cultured as a monolayer in 75 cm^2 culture flasks at 37°C in 5% CO_2. The ZR-75 cell line was grown in RPMI complete medium. All other cell cultures were grown in DMEM complete medium. Subculturing cells was necessary once a flask was confluent. To do this, the medium was removed; cells were washed with 5mL of PBS after which 4-5mL of Trypsin was added. The flask was incubated at 37°C in 5% CO_2 until cells detached from the flask (normally 5 minutes). To inactivate the enzymatic activity 5 mL of growth medium was added to the cell suspension using a pipette. The cells were then centrifuged at 1,000 rpm for 5 minutes, supernatant was removed and cells were resuspended in fresh medium. At this point a fifth of this suspension was transferred to a fresh flask and incubated at 37°C in 5% CO_2 as normal.
For the purposes of gene expression analysis and protein expression, all cells were grown to ~40% confluency. In addition to this, cell lines were not passaged any greater than 20 times from time of purchase to avoid substantial phenotypic changes.

2.2.2 Isolation of breast epithelial cells from Reduction Mammoplasties

Samples from reduction mammoplasties were selected by a pathologist and were placed in complete medium containing 0.5mL Penicillin Streptomycin (10,000U/mL of Penicillin and 10mg/mL of Streptomycin), and 0.5mL Fungizone (250 UG/mL).
All fat was removed and the fibrous tissue was finely chopped avoiding crushing using a sterile scalpel. Approximately 1400mm^2 was chopped, which was divided equally between four 50mL plastic tubes each containing 20mL complete medium, 0.5mL Penicillin streptomycin solution (containing 10,000U/mL of Penicillin and 10mg/mL of Streptomycin), 0.5mL Fungizone (250 UG/mL), 2mL collagenase (433U/mg), and 0.5mL hyaluronidase (5mg/mL) and incubated overnight at 37°C on a rocker. Following digestion organoids were isolated (for overview, see Figure 2.2). Organoids are individual breast glands and comprise an epithelial and myoepithelial bilayer (see Figure 2.1).

Figure 2.1 Diagram of breast epithelial cell

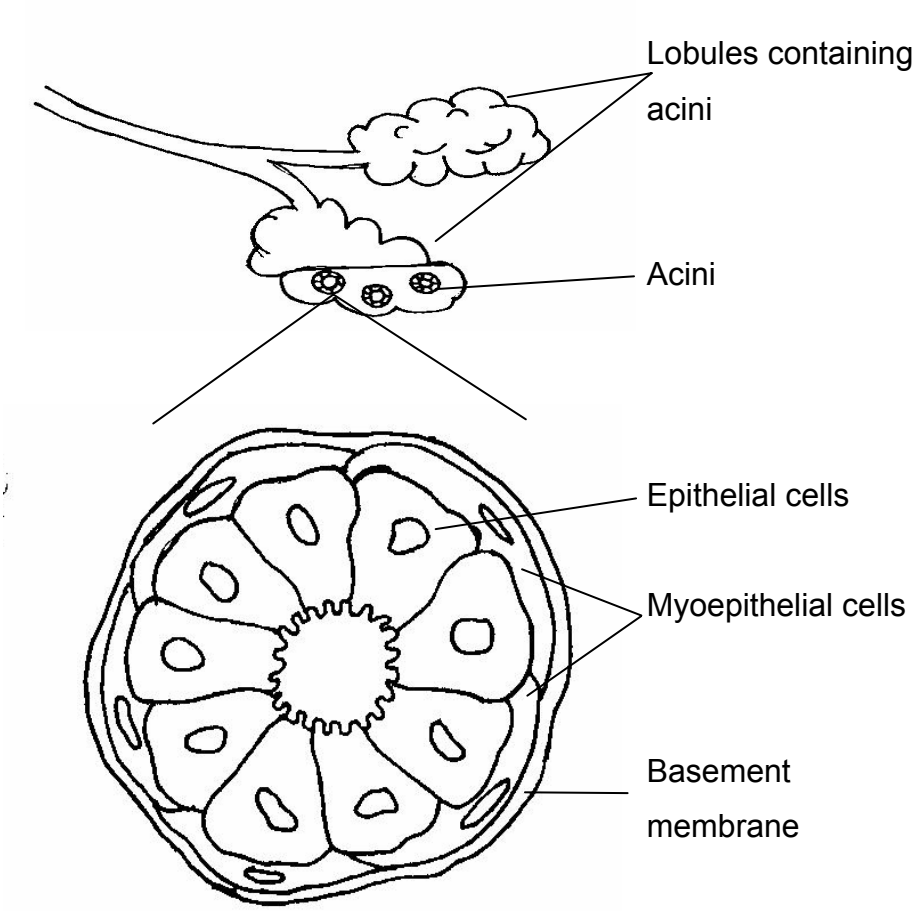
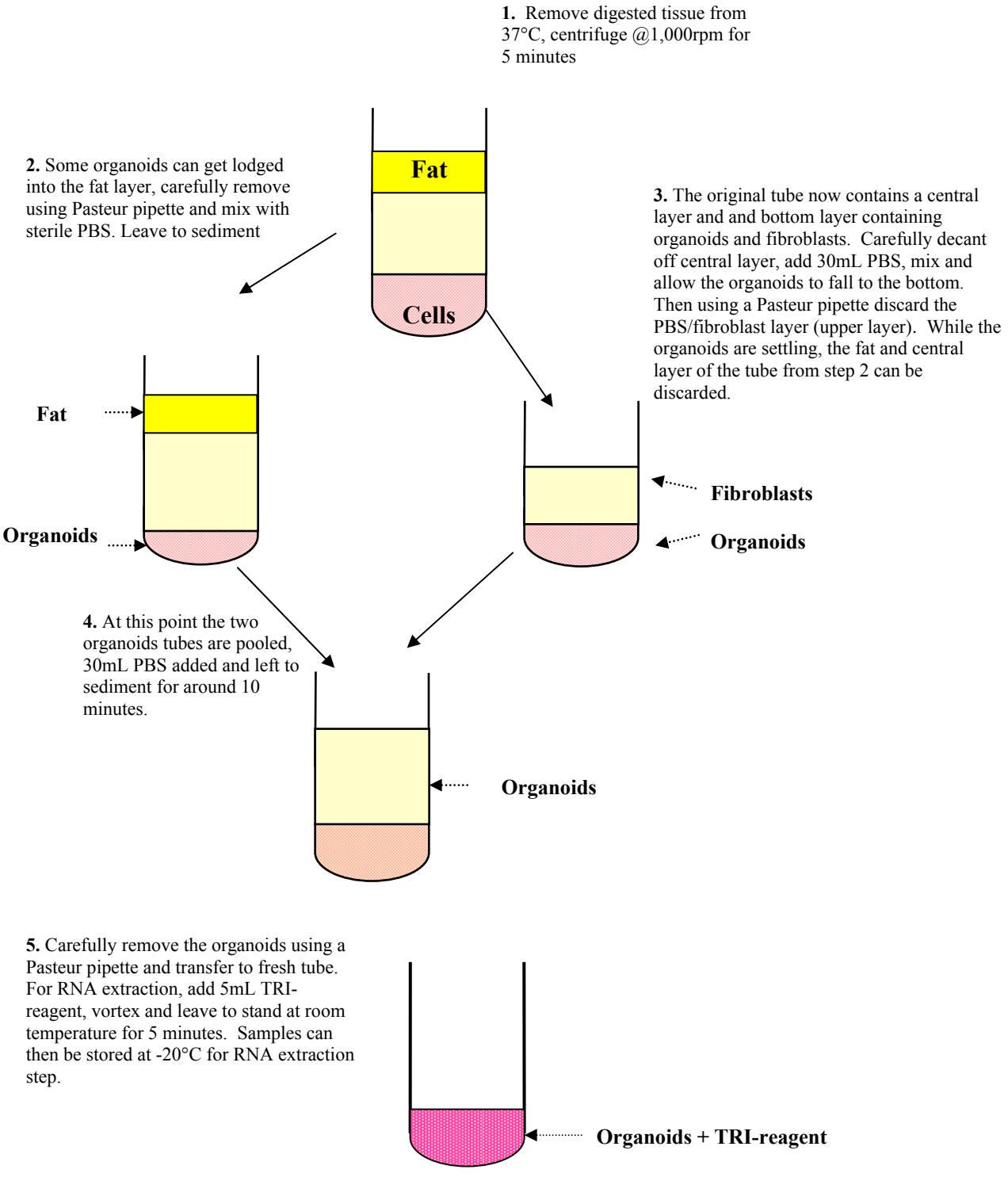


Figure 2.2 Overview of organoids extraction



2.2.3 RNA Extraction

2.2.3.1 Cell lines, organoids and frozen tissues

RNA was extracted from cell line pellets/organoids/frozen tissues using a double TRI-reagent extraction. For frozen tissue, 10 × 4μM thick sections were cut from the tumour blocks. The cell line pellets/organoids/frozen sections were first lysed and digested in 1mL of TRI-reagent and allowed to stand at room temperature for 5 minutes. 200μL chloroform was added to each sample, mixed thoroughly, stood at room temperature for 3 minutes, and centrifuged @ 16,467g for 15 minutes at 4°C. The aqueous layer was transferred to a fresh eppendorf, with subsequent addition of 500μL TRI-reagent and 100μL chloroform. Samples were mixed, stood at room temperature for 5 minutes, and centrifuged at 16,467g for 15 minutes at 4°C. The aqueous layer was again transferred to a fresh eppendorf, and 1μL 1mg/μL glycogen and 500μL isopropanol added. Samples were stood at room temperature for 10 minutes and centrifuged @16,467g for 10 minutes at 4°C. The supernatant was carefully discarded and the cell pellet washed with 1mL 70% ethanol, and centrifuged at 16,467g for 15 minutes at 4°C. The supernatant was removed and the pellet air-dried for 5 minutes before resuspending in 100μl of sterile molecular biology grade H_2O. To remove any contaminating genomic DNA, samples were treated with 0.1 volume 10 × TURBO DNase buffer and 1μL DNase for 20 minutes at 37 °C. To inactivate the reaction, 0.1 × volume DNase inactivation reagent was added, incubated for 2 minutes at room temperature, centrifuged @ 9,744 rpm, after which the supernatant was transferred to a fresh tube. Samples were quantitated using a NanoDrop® ND-1000 UV-Vis Spectrophotometer.

2.2.3.2 Formalin Fixed Paraffin Embedded tissue

Ten × 4μM thick sections were cut from the tumour blocks and put in sterile eppendorfs. To dewax the sections, 1mL of xylene was added, vortexed, incubated for 5 minutes at room temperature and centrifuged for 5 minutes at 10,000 rpm. The supernatant was discarded. This dewaxing step was repeated. The tissue was twice rehydrated in 2mL 99% EtOH,

vortexed, incubated at room temperature for 2 minutes, and centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded taking care not to dislodge the tissue. A further rehydration step was performed, this time using 95% EtOH. After the supernatant was removed, the tissue was air-dried for 5-10 minutes. Tissue was resuspended in 200µL extraction buffer (0.01M Tris pH 8, 0.1M EDTA, 2% SDS), and 10µL, 100µL or 200µL of 10mg/mL Proteinase K solution (final concentration of PK was 0.5, 5, 10mg/mL). Samples were incubated at either 37ºC or 50 ºC for 24, 48 and 120 hours. Incubation temperatures, durations, and Proteinase K concentrations were varied to determine the conditions for optimal RNA extraction (see Table 2.2). For optimisation purposes all reactions were carried out in triplicate. After incubation with PK, RNA extraction proceeded with the phenol:chloroform steps outlined above.

Table 2.2 Summary of Proteinase K incubation conditions

PK concentration mg/mL	Temperature (ºC)	Incubation time (hours)
0.5	37/50	24/48/120
5	37/50	24/48/120
10	37/50	24/48/120

2.2.4 Primer design, validation and optimisation

Complete cDNA sequences for candidate genes were retrieved from NCBI Nucleotide database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi).

2.2.4.1 Primer design

Oligonucleotide primer pairs were designed for the nine candidate genes and three housekeeping genes for use in real time qPCR (see Table 2.3). Nucleotide sequences obtained from the NCBI Nucleotide database were then used to design primers using the Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers 22-25 base pairs long were designed to give a product of between 90-105 bps. They were selected from regions close to the polyadenylation site to minimise limitation in RT efficiency. Primer T_m criteria were set between 64-67°C with a GC% of 40-55%. Primers were selected for minimum self-complementarities. The maximum poly-X nucleotide run was also kept to 2 if possible. All primers were designed for use at 65mM sodium concentration.

2.2.4.2 Primer validation

To test for specificity NCBI Blast (http://www.ncbi.nlm.nih.gov/BLAST/) was used to look for short, nearly exact matches. Both forward and reverse primer sequences were tested. Where results for unknown genes or genomic clones were found, checks were made to ensure that these referred to the gene of interest.

The amplification products of the primers were then tested for secondary structures using DNA mfold (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/) with a folding temperature of 55°C and the ionic conditions set as 65 mM Na^+ and 3 mM Mg^{2+}. The dG of the structures was used to determine the likelihood of these secondary structures forming. Those with a positive dG will never form while those with small negative dG are unlikely to form. Only secondary structures with a negative dG of greater than –2 were considered significant, requiring redesign of the primers.

Finally primer sequences were checked using the Vector NTI 7 (Informax, Inc.). This tested for hairpin loops, primer dimers and duplex formation. Again the dG value determined the likelihood of formation of these structures. Reaction conditions were set as before.

Table 2.3 Summary of primer sequences and respective annealing temperatures

Gene Name	Forward Primer 5'-3'	Reverse Primer 5'-3'	Tm (°C)	Amplic-on size
AKAP1	AACATTGTCCTCTCCAGAAAGTCCT	TGCGAAGAGAACCCATAGTTCCAT	60	105
DDB2	CAAGCAGAGGTGGTGATTTG	AAAAGTGTCCCAGTCCCACA	64	104
APRIL	ATGAAGAGGAGGAAGAAGGTGGGAAA	GCAGGTCATCTGGGGTCTTAATCATC	64	94
NCOA3	CAACTCCAAGGCACACTGT	TTCCTGAGAATTTAAATAT	63	108
C/EBPα	CCTTGTGCCTTGGAAATGCAA	GAAGGAGGCAGGAAACCTCCAA	67	103
Rarres3	AAAAGCAACAGCCTGAAGCA	GCTGGAGGCATGGGGAGGCTCAT	60	100
RBBP4	CTCGAAAATCTTGACACCTGACTTT	GGAAAGAGGAACGTGTTGTTTTGA	62	104
Granulin	CTAGCACCTCCCCCTAACCA	CTGACAGGGAAGGCCTTAGA	60	99
TGF-βI	TGGACAGACCCTGGAAACTC	TGAACAGGGTCCCGTACCT	65	102
PBGD	AGATGAGAGTGATTCGCGTGGGTA	AGGGTACGAGGCTTTCAATGTTGC	60	92
TFRC	TTGTAATGGGAACTGCCTCTTTCC	ACCTTCAGCAGAGACCAGCCCTTA	57	104
HPRT1	GCAGACTTTGCTTTCCTTGGTCAG	GTCTGGCTTATATCCAACACTTCGTG	57	103

HPRT1 - hypoxanthine phosphoribosyltransferase 1, TFRC - Transferrin receptor, PBGD - Porphobilinogen deaminase, selected as housekeeping genes.

2.2.5 RT-PCR

2.2.5.1 Reverse Transcription

3 µg total RNA was reverse transcribed using Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT). The reaction consisted of 29.15µL of master mix (see Table 2.4) and made up to 50µL with sterile molecular biology grade water. The reaction was incubated at 42°C for 15 minutes, followed by 99°C for 5 minutes. Negative reactions were undertaken to ensure no genomic DNA contamination.

Table 2.4 Reverse transcription master mix

Reagent	Volume (µL)
Magnesium Chloride ($MgCl_2$), 25mM	12
Reverse Transcription 10 × Buffer	6
dNTP Mix, 10mM	6
Recombinant RNasin® Ribonuclease inhibitor, 40 U/µL	1.5
Random primers	1.5
AMV-RT, 24U/ µL	2.15
Total	10.75

2.2.5.2 Polymerase chain reaction (PCR)

1 µL of cDNA was added to aliquots of PCR master mix (see Table 2.5) to make a final reaction volume of 50 µL. Reactions were performed in the Perkins Elmer GeneAmp® 2200. The conditions were: an initial incubation at 94 °C for five minutes, followed by 30 cycles at 94 °C (30 seconds), annealing temperature specific for the primer pair (see Table 3.1) (30 seconds), 72 °C (30 seconds), and 10 min final extension at 72 °C. PCR products were run on 3% agarose gels and visualised using ethidium bromide staining.

Table 2.5 Polymerase chain reaction master mix

Reagent	Volume (µL)
Sterile molecular biology grade water	40
10 X Alex Jeffrey's buffer	5
Forward primer 10 pM/ µl)	1
Reverse primer (10 pM/ µl)	1
Taq DNA polymerase (diluted 1:10 with H_2O)	2
Total	49

2.2.6 Real time quantitative RT-PCR

2.2.6.1 SYBR Green

Real time qPCR reactions were carried out using the Platinum® SYBR® Green qPCR SuperMix UDG kit and the Mx4000® Multiplex Quantitative PCR System. 1 µL of cDNA was added to the SYBR green master mix (see Table 2.6) for a final reaction volume of 25 µL. Each primer pair was tested with a logarithmic dilution of a cDNA mix to generate a linear standard curve, which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$). The relevant abundance of each transcript was calculated based on PCR efficiency and cycle number at which the fluorescence crossed a cycle threshold (Ct). The generation of specific PCR products was confirmed by melting curve analysis.

To normalise the target gene expression, three housekeeping genes were tested (HPRT1, TFRC and PBGD).

The expression of nine target genes and three housekeeping genes were tested in all cell lines, organoids from nine reduction mammoplasties, and 36 tumour samples. All experiments were carried out in triplicate. Target gene expression was represented as a ratio of target gene transcripts/house-keeping gene transcripts (taken as the average of HPRT1, PGBD, and TFRC genes).

Table 2.6 SYBR green master mix

Reagent	Volume (µL)
Platinum® SYBR® Green	12.5
Internal Reference Dye, 1:10	0.5
Forward primer 10 pM/ µl)	0.5
Reverse primer 10 pM/ µl)	0.5
Sterile molecular biology grade water	10
Total	24

2.2.6.2 Taqman gene expression assay

GeneAmp® Fast PCR Master Mix (2x) and QuantumRNA™ 18S Internal Standards were used to carry out 18S expression in cell lines, organoids, and all tumour cases. 4µL of cDNA was added to the Taqman Fast Universal Master Mix (see Table 2.6), for a final reaction volume of 10µL. Each run included a 7-fold dilution of a cDNA standard from which a linear standard curve was generated. The relative abundance of 18S in each sample was then calculated from the PCR efficiency and the cycle number at which the fluorescence crossed the cycle threshold (Ct). All experiments were carried out in triplicate.

Table 2.7 Taqman gene expression master mix

Reagent	Volume (µL)
Taqman Fast Universal Master Mix	5.0
18S probes	0.5
H_2O	0.5
Total	6.0

2.2.7 Western Blotting

Monoclonal antibody NCOA3 and polyclonal antibody RARRES3 were used in semi-quantitative analysis of protein expression in the breast cell lines HBL-100, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T47-D, and three organoid specimens.

2.2.7.1 Sample Preparation

Cells were harvested when 40% confluent using trypsin-EDTA. After inhibition of trypsin activity with complete medium, cells were spun at 1,000 rpm for 5 minutes. Supernatant was discarded and cell pellet was washed in cold sterile PBS. The cell pellet was then resuspended in 300 µL of lysis buffer and 3µL protease inhibitor, mixed thoroughly, and stood on ice for 10 minutes. To ensure lysis, cells were passed through a 23 gauge hypodermic needle 15 times. The lysed cells were then centrifuged at 13,000 rpm for 1 minute to remove cell debris and the resulting supernatant transferred to a fresh sterile eppendorf. Samples were stored at -20ºC until subsequent analyses.

2.2.7.2 Protein quantification

Protein concentration in each sample was determined using Bio-Rad protein assay reagent. Each sample was prepared as follows: 5 µL of protein lysate, 795 µL H_2O and 200 µL of protein assay reagent. Samples were mixed thoroughly and developed in dark for 5 minutes. Absorbance at 595 nm was measured on a spectrophotometer. Absolute protein concentration in samples was then calculated from a standard curve, constructed from absorbance against protein concentration for a series of bovine serum albumin protein standards.

2.2.7.3 Gel preparation

Stacking and resolving SDS-page monomer solutions were prepared, the concentrations depending on the size of the protein being analysed: 8% SDS-PAGE gels for NCOA3 (160 kDa), and 12% SDS-PAGE gels for RARRES3 (37 kDa) and beta-Actin (43kDa). (see Table 2.8).

Table 2.8 Summary of gel preparation

	8%	12%
Reagent	**Volume**	
H_2O	4.6 mL	3.3 mL
30% Acrylamide/Bis	2.7 mL	4.0 mL
Gel buffer*	2.5 mL	2.5 mL
10% w/v SDS	0.1 mL	0.1 mL

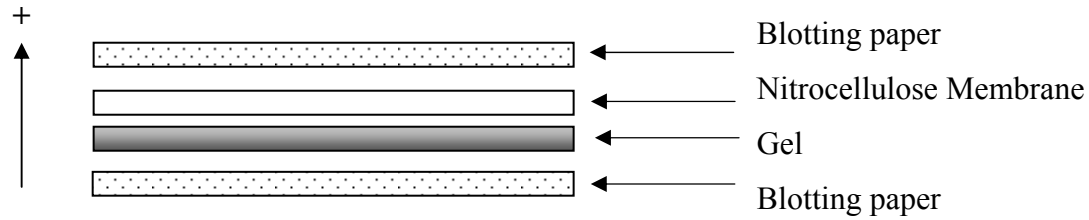
*Resolving gel buffer – 1.5 mM Tris-HCL pH 8.8, stacking gel – 0.5 mM Tris-HCL pH 6.8

Immediately prior to pouring the gel, 50 µL 10% APS and 5 µL TEMED were added to the resolving gel monomer solution to initiate polymerisation. After the resolving gel had set, 50 µL 10% APS and 5 µL TEMED were added to the stacking gel and this was put on top of the resolving gel. This again was allowed to set. Twenty-five µg of each protein lysate was mixed with 3 µL of loading buffer, denatured at 99ºC for 5 minutes, cooled on ice and then loaded into each well. 10µL BioRad Precision Plus Dual Color protein weight marker was also loaded into a separate well. The gel was then run at 80 V until the samples reached the resolving gel, then at 140 V until the coloured marker had almost run off the gel. BioRad® mini-gel apparatus was used for the running of the gel.

2.2.7.4 Protein transfer and blocking

The gel was removed and washed in transfer buffer for 5 minutes. One piece of pre-wet nitrocellulose membrane and two pieces of Whatman 3 mm filter paper equal in size to the gel were soaked in transfer buffer with methanol. The nitrocellulose membrane was placed on top of the gel and size markers clearly marked on the membrane using a ball point Biro pen. The gel and membrane were then sandwiched between the pieces of filter paper as shown in Figure 2.3. Air bubbles were removed. The gel and papers were transferred into a cassette and protein electrophoresed onto the nitrocellulose membrane for 1.5 hours at 100V. Again, BioRad® mini-transfer equipment was used.

Figure 2.3 Schematic overview of protein transfer setup



2.2.7.5 Western analysis

After transfer, the nitrocellulose was removed from the transfer apparatus, washed in washing buffer to remove residual SDS, and soaked for 1 hour in blocking solution. It was washed 3 times, for 5 minutes each, in washing buffer. The nitrocellulose membrane was then probed with primary antibody overnight. All antibodies were diluted in blocking solution: NCOA3 (1:1,000), RARRES3 (1:500), and beta-Actin (1:1,000). The membranes were washed 3 times as above and incubated with secondary antibody, anti-mouse horseradish peroxidise linked whole antibody 1:2,000 in blocking solution, for 30 minutes (NCOA3) or 60 minutes (RARRES3). An Amersham Biosciences ECL detection kit was used for visualisation according to manufacturer's instructions. X-ray film was used for the detection of resulting chemiluminescence, with exposure time of 30 seconds, 2 minutes and 5 minutes before development. In the case of RARRES3, it was necessary to strip the blot to remove all antibodies for subsequent beta-Actin analysis. This was done by soaking the blot in a 1:1 solution of 30%H_2O_2/PBS for 30 minutes, followed by two washes of washing buffer, before detection with the beta-Actin antibody could be carried out.

2.2.8 Immunohistochemistry

2.2.8.1 Microtomy

FFPE tissue blocks were placed on ice for at least 15 minutes. Sections of 4µm thickness were cut using a microtome, floated out on a water bath at 45ºC and placed onto vectabond coated slides. Slides were dried overnight at 37 ºC before starting the staining process.

2.2.8.2 Antigen Retrieval Optimisation

All antibodies were optimised to ascertain the optimal antigen retrieval conditions using a microwave or pressure cooker using various incubation temperatures, and duration.
To expose an antigen using the pressure cooker, 250mL of 1 x DAKO antigen retrieval buffer was placed in the removable containers of the Stainless Steel pressure cooker model Pascal. The containers were then placed inside the pressure cooker, brought to 80ºC followed by the insertion of slides within metal slide racks. The lid was locked into position and the pressure was allowed to rise. When the optimum temperature was attained, the slides were treated for different period (see Table 2.9) for a summary of pressure cooker variables used in optimisation). The slides were then cooled to 90ºC, then placed under running water and rinsed in distilled water.
For antigen retrieval using a microwave, slides were placed in a polystyrene container with 500mL 1 x TE buffer pH 9 and heated on full power for 20 minutes. After heating the slides were left to cool in the buffer for 45 minutes.

Table 2.9 Summary of Pressure Cooker Optimisation Variables

Temperature ºC	**Time**		
120	30	45	60
123	30	45	60
124	30	45	60
125	30	45	60

2.2.8.3 Immunohistochemical Protocol

The immunohistochemical technique was optimised for various parameters including, antigen retrieval method, primary antibody concentrations, and incubation temperature and duration. These variables are summarised in Table 2.10. Visualisation methods were confirmed with information from the antibody data sheets and their cited references.

Table 2.10 Summary of antigen retrieval methods and primary antibody dilutions attempted during NCOA3 optimisation

Optimisation Process	Primary Antibody NCOA3
Antigen Retrieval: Pressure cooker 120, 123, 125 ºC for 30,45 or 60 seconds Antigen Retrieval: Microwaving 20 minutes at full power	Primary Antibody dilutions 1:20, 1:40, 1:100, 1:200

The results were reviewed by 2 individuals (Prof. R.A. Walker and I), and the conditions yielding the optimum staining pattern and least background staining were chosen. The final antigen retrieval methods and dilutions for NCOA3 are shown in Table 2.11. The immunohistochemical procedure is outlined in Figure 2.4. In each IHC experiment, a positive control, and negative controls were included.

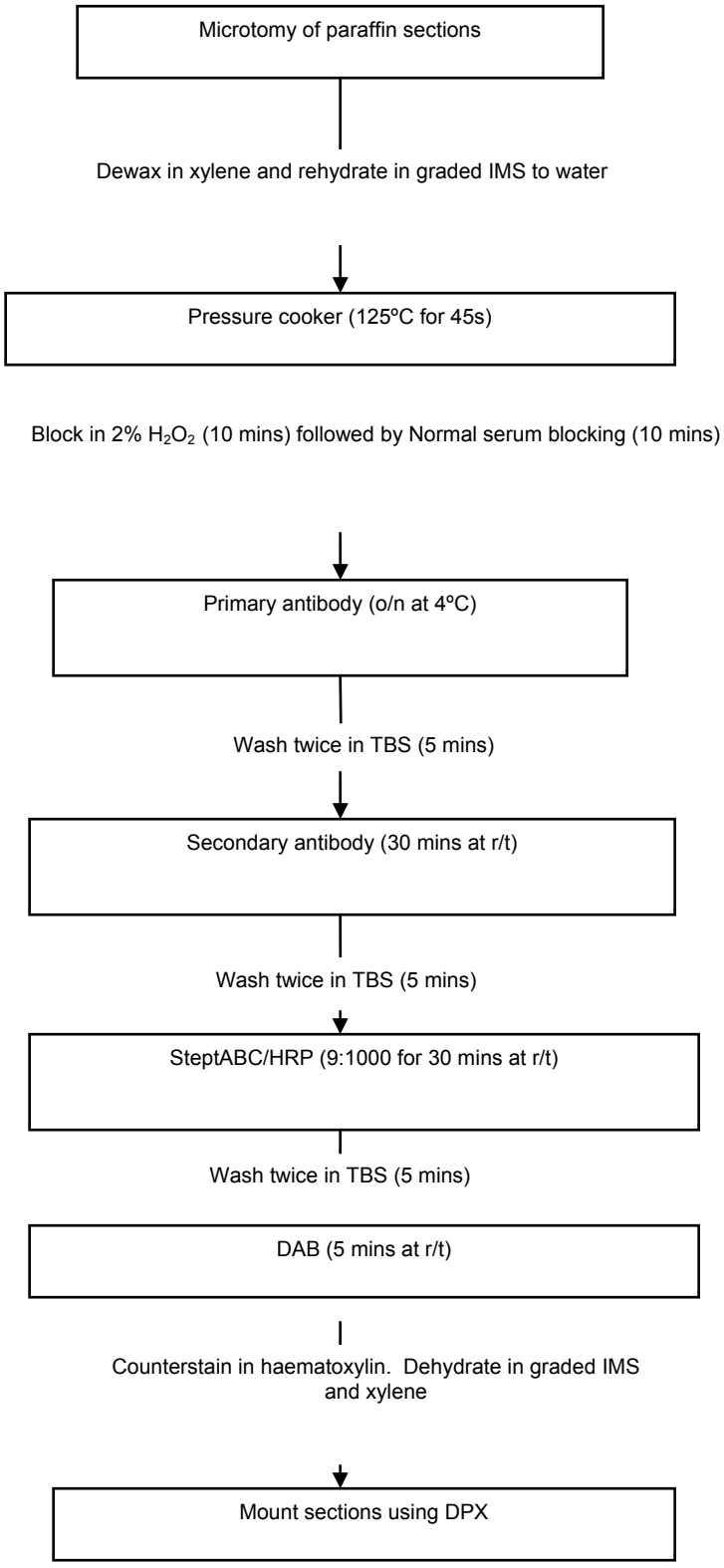
2.2.8.4 Grading of Staining Intensity

Specimens were assessed and each staining extent was graded as the percentage of positively stained cells, and categorised as negative (<1% staining), low (1-5% staining), moderate (5-50% staining), and high (>50% staining) in comparison to the positive control.

Table 2.11 Summary of final Immunohistochemical methods and dilutions

Antibody	Antigen retrieval method	Primary antibody	Secondary Antibody	Tertiary Reagent	Visualisation method
NCOA3	Pressure cooker 125ºC for 45 seconds	1:40	1:400 RαmBt	SteptABC/HRP 9:1000	DAB

Figure 2.4 Flow diagram showing the immunohistochemical protocol



2.2.9 Statistics

Real-time quantitative PCR data was first tested to check if it had a normal distribution. All datasets which followed a normal distribution were then tested for significance using a 1-tailed-independent t-test in the case of the FFPE gene expression data, and analysis of variance (ANOVA) with the Bonferroni post-hoc test for the snap frozen gene expression data. Results for immunohistochemistry were correlated with age and clinical prognostic markers using the Pearson's χ-squared exact test. All analyses were performed using SPSS Version 14.

Chapter 3. Results

3.1 Selection of target genes

Microarray analysis was used to compare cDNA isolated from two breast cancers from women ≤35 years old with the non-tumourigenic HBL-100 cell line and normal organoids isolated from reduction mammoplasty. SAM (Significance Analysis of Microarrays) was used to compare gene expression profiles between the 2 tumours and HBL-100, where it identified 470 up-regulated and 285 down regulated genes in common between the 2 tumours. When SAM analysis was carried out to compare the 2 tumours to the normal organoids, 69 genes were found to be up regulated and 372 were down regulated. There was significant overlap between these datasets.

HBL-100, although non-tumourigenic, shows a high level of invasion (Gordon *et al.*, 2003) therefore it was felt that comparison between cancer and organoid RNA was more likely to reveal true tumour-specific changes. It was therefore decided to focus on the validation of candidate gene targets identified from comparison to the organoid sample. The most significant fold changes were ranked in order and bioinformatic analysis was used to select suitable candidate genes for further study. This involved investigating the function of each gene, and/or its known relevance to cancer using a literature search (Pubmed). Following a literature search of the dataset containing genes that were up regulated, nine genes were selected for further study (see Table 3.1). None of the nine genes had been identified in previous published microarray investigations (see Table 1.2). All of these genes were up regulated in tumours compared to the organoid, and 7 were also up regulated relative to HBL-100. The functions of each of these genes will be discussed in the form of a literature review, with particular emphasis on their potential role in cancers.

Table 3.1 Target genes from microarray data that were selected for further study

Gene Name	Microarray fold change		Gene location	Presumed function
	Tumour vs. organoids	Tumour vs. HBL-100		
A kinase anchor protein 1 (AKAP1)	3.11	3.83	17q21-q23	Intracellular loclisation of PKa and PP1
Acidic protein rich in leucines (APRIL)	2.23	2.52	9q22.32	Regulating ribosomal RNA biosynthesis
CCAAT enhancer binding protein alpha (C/EBPα)	2.96	6.55	19q13.1	Transcription factor
Damage specific DNA binding protein 2 (DDB2)	2.30	=	11p12-p11	DNA repair via NER
Granulin	4.09	2.70	17q21.32	Autocrine growth factor
Nuclear receptor coactivator 3 (NCOA3)	5.33	3.01	20q12	Enhances transcriptional activator functions of nuclear receptors
Retinoic acid receptor responder 3 (RARRES3)	3.49	5.85	11q23	Cell differentiation and growth suppression
Retinolastoma binding protein 4 (RBBP4)	2.04	=	1p35.1	Involved in histone acetylation and chromatin assembly
Transforming growth factor beta induced (TGFβI)	3.53	2.17	5q31	Adhesion protein

= gene expression equal to that of HBL-100

3.1.1 A-kinase Anchoring Protein 1

A-kinase anchoring protein 1 (AKAP1), located at 17q21-q23, is also known as AKAP149, and is a member of a family of anchoring proteins (AKAPs) that act as scaffolding proteins to bring enzymes important for cell signalling such as cyclic-AMP-dependent protein kinase (PKA) and calcium- and phospholipid-dependent kinase (PKC) within reach of their targets (Feliciello *et al.*, 2001). It was first identified in the mitochondrion (Chen *et al.*, 1997b; Ginsberg *et al.*, 2003) and endoplasmic reticulum-nuclear envelope (Steen & Collas, 2001), where it appears to have roles in RNA processing (Trendelenburg *et al.*, 1996), and binding of protein phosphatase 1 (PP1) to the nuclear envelope during its reassembly after mitosis

(Steen *et al.*, 2003; Steen *et al.*, 2000). So far no reports have suggested that AKAP1 is associated with carcinogenesis.

3.1.2 Acidic Protein Rich in Leucines

Mapped at 9q22.32, acidic protein rich in leucines (APRIL) is a member of the acidic nuclear phosphoprotein family, which help to modulate cellular signalling and gene expression to regulate the morphology and dynamics of the cytoskeleton, cell adhesion, neuronal development, or cerebellar morphogenesis (Matilla & Radrizzani, 2005). APRIL is believed to participate in the progression of G1 to the S phase of the cell cycle, as well as in transduction that directs nucleoli activities by regulating ribosomal RNA biosynthesis (Sun *et al.*, 2001). High expression of APRIL has been found in chronic myeloid leukaemic and Burkitt lymphoma cell lines (Mencinger *et al.*, 1998), but not in any epithelial cancers to date.

3.1.3 CCAAT enhancer binding protein alpha

CCAAT/enhancer binding proteins (C/EBPs) are structurally related transcription factors that play a role in normal tissue development, cellular proliferation and differentiation (Lekstrom-Himes & Xanthopoulos, 1998; Robinson *et al.*, 1998; Seagroves *et al.*, 1998). C/EBPα, mapped to 19q13.1, the earliest member of the C/EBP family to be identified, encodes a protein that has an important role in the regulation of mitotic growth arrest and differentiation in numerous cell types, including preadipocytes (Cao *et al.*, 1991) myelomonocytic cells (Scott *et al.*, 1992), hepatocytes and pneumocytes (Flodby *et al.*, 1996), and keratinocytes (Oh & Smart, 1998). To date, C/EBPα down-regulation has only been observed in breast (Gery *et al.*, 2005) and lung tumours (Halmos *et al.*, 2004) as well as in leukaemias (Pabst *et al.*, 2006), where it appears to function as a tumour suppressor. In their study, Gery *et al.* saw a down-regulation of the C/EBPα protein in 83% of 24 primary breast cancers which was independent of their ER status (Gery *et al.*, 2005). Several mechanisms have been put forward to explain the putative tumour suppressor function of C/EBPα.

These include: the ability of C/EBPα to regulate p21 and retinoblastoma (Rb) family or proteins (Chen *et al.*, 1996; Timchenko *et al.*, 1996); form complexes with cdk2 and cdk 4 thereby inhibiting their interactions with cyclin E and cyclin D respectively (Wang *et al.*, 2001); associate itself directly with E2F in so doing blocking transcriptional activation of its

target genes e.g. c-myc (Slomiany *et al.*, 2000); and to interact with the SWI/SNF chromatin remodelling complex (Muller *et al.*, 2004).

3.1.4 Damage Specific DNA Binding Protein 2

Mapped to 11p12-p11, damage-specific DNA binding protein 2 (DDB2) encodes a 48kDa protein which is a component of the damage-specific DNA-binding protein (DDB) complex that also includes damage-specific binding protein 1 (DDB1) ((Dualan *et al.*, 1995) (Takao *et al.*, 1993). The DDB complex plays an important role in nucleotide excision repair (NER) where DDB2 plays a specific role in detection of cyclobutane pyrimidine dimers (CPDs) and binding of the complex to DNA (Li *et al.*, 2006; Tang *et al.*, 2000). Mutations in DDB2 cause Xeroderma pigmentosum group E (Itoh *et al.*, 1999; Nichols *et al.*, 2000), a disease characterised by defective nucleotide excision repair (NER). DDB2 has not been strongly linked to human cancers to date. However, inactivation of DDB2 expression has been found to result in the formation of tumours in mice (Yoon *et al.*, 2005), while overexpression of DDB2 appears to protect mice from the carcinogenic effects of UV irradiation (Alekseev *et al.*, 2005).

Hwang *et al.* (1999) showed that DDB2 expression may depend on basal TP53 expression. This is increased further after DNA damage in a TP53-dependent manner. In addition, a subsequent study showed that DDB2 can down-regulate TP53, while a reduction in DDB2 led to a rapid fall in TP53-dependent apoptosis (Itoh *et al.*, 2003). Using mouse knock-out models, the same group showed that DDB2 enhanced lung and mammary adenocarcinoma, and regulated apoptosis (Itoh *et al.*, 2007). BRCA1 can also induce DDB2 mRNA expression in a TP53-independent manner (Hartman & Ford, 2002). More recently, DDB2 has been shown to influence cell cycle by interacting with cell cycle regulated genes such as E2F (Kaufmann *et al.*, 2007). A more relevant study to breast cancer found that the c-myc oncogene down regulated TP53 target genes such as DDB2 (Ceballos *et al.*, 2005).

3.1.5 Granulin

Granulin, also known as progranulin or PC-cell derived growth factor, is an 88 kDa growth factor that is the precursor of a novel family of growth modulating polypeptides characterized by a unique and highly conserved cysteine-rich motif (12 cysteines). The biological functions of granulin are varied, but appear to be connected to processes that require fast cell turnover, such as wound healing and repair (He & Bateman, 2003), embryonic development

(Daniel *et al.*, 2003), as well as cellular processes vital to cancer progression such as proliferation, invasion, and metastasis (Cheung *et al.*, 2004; Tangkeangsirisin & Serrero, 2004). Over-expression of granulin has been found in several tumour cell lines and/or tumour tissues at the mRNA level in breast (Lu & Serrero, 2000), ovarian cancers (Davidson *et al.*, 2004), gliomas (Markert *et al.*, 2001), and acute myeloid leukaemia (Virtaneva *et al.*, 2001), and at the protein level in ovarian (Jones *et al.*, 2003), renal (Donald *et al.*, 2001), breast (Tangkeangsirisin & Serrero, 2004), endometrial (Jones *et al.*, 2006) cancers, and at both mRNA and protein levels in hepatocellular cancer (Cheung *et al.*, 2004). Transfection of breast cancer cells with granulin was reported to mediate the mitogenic effect of oestradiol (Lu & Serrero, 2001), stimulate anchorage-independent growth, angiogenesis, and metastasis via stimulation of MMP, and VEGF expression (Tangkeangsirisin & Serrero, 2004), and render cells resistant to tamoxifen (Tangkeangsirisin *et al.*, 2004) thereby promoting tumour growth and survivability. Protein analysis in a cohort of paraffin embedded breast biopsies found a correlation between granulin expression and clinicopathological parameters such as high grade, high proliferation index, and high p53 expression (Serrero & Ioffe, 2003,) providing further evidence for an important role for granulin in cancers. Similarly, granulin has been associated with proliferation, invasion and metastasis in hepatocellular (Cheung *et al.*, 2004), bladder (Monami *et al.*, 2006), and ovarian (Pizarro *et al.*, 2007) cancers, as well as with p53 expression in hepatocellular carcinomas (Cheung *et al.*, 2006). Oestrogen has been found to regulate granulin in endometrial (Jones *et al.*, 2006) and breast (Lu & Serrero, 2000) cancer cells.

3.1.6 Nuclear Receptor Coactivator 3

Nuclear Receptor Coactivator 3 (NCOA3), localised to chromosomal region 20q12 (Anzick *et al.*, 1997) is a member of the p160/steroid receptor coactivator family which includes NCOA1 and NCOA2. NCOA3 was first identified as Amplified in Breast Cancer 1 (AIB1) and is also known as ACTR (Chen *et al.*, 1997a), TRAM1 (Takeshita 1997), RAC3 (Li *et al.*, 1997) and SRC3 (Suen *et al.*, 1998). Like other family members NCOA3 associates with nuclear receptors, such as the ER and PgR (Han *et al.*, 2006), and thyroid receptor (Takeshita *et al.*, 1997) as well as with several transcription factors, including E2F1 (Louie *et al.*, 2004), signal transducer and activator of transcription (STAT) (Arimura *et al.*, 2004), nuclear factor-kappa B (NFκB) (Werbajh *et al.*, 2000), and activator protein-1 (AP-1) (Lee *et al.*, 1998; Yan *et al.*, 2006). Binding of NCOA3 to these transcription factors recruits chromatin

modification factors such as acetyltransferases (CBP and p300) and methyltransferases (CARM1 and PRMT1), leading to a modification of the chromatin structure and activation of transcription of their target genes (Chen *et al.*, 1999; McKenna & O'Malley, 2002).

NCOA3 overexpression has been observed in several different cancers, including breast (Anzick *et al.*, 1997), gastric (Ghadimi *et al.*, 1999), prostate (Gnanapragasam *et al.*, 2001), pancreatic (Henke *et al.*, 2004), melanoma (Rangel *et al.*, 2006), endometrial (Kershah *et al.*, 2004), endocervical (Hirai *et al.*, 2004) and colorectal (Xie *et al.*, 2005) carcinomas. Overexpression of NCOA3 was observed in ~60% of primary human breast tumours as a result of transcriptional up-regulation or gene amplification (Anzick *et al.*, 2003), (List *et al.*, 2001), (Reiter *et al.*, 2001). A small number of studies have explored the relationship between NCOA3 expression and diagnostic and prognostic markers for breast cancer, such as oestrogen receptor, progesterone receptor, p53, and human epidermal growth factor receptor 2 (HER-2/neu). One such study pointed towards a correlation between NCOA3 expression and the presence of ER receptor (Iwase *et al.*, 2003), while others indicated a relationship between NCOA3 expression and ER/PgR negative and p53/HER-2/neu positive breast cancers (Bouras *et al.*, 2001; Kirkegaard *et al.*, 2007; Osborne *et al.*, 2003). The study carried out by Iwase *et al.* also showed that patients with NCOA3 nuclear expression tended to respond better to hormonal therapy. In contrast, two other studies found an association between NCOA3 expression and tamoxifen resistance (Dihge *et al.*, 2007; Osborne *et al.*, 2003). In addition, a recent study found a relationship between the presence of NCOA3 in HER-2 positive cancers and early relapse and death in tamoxifen-treated breast cancers (Kirkegaard *et al.*, 2007).

The precise way by which NCOA3 exerts its cancer promoting actions is not clear. In hormone sensitive breast cancers, NCOA3 appears to enhance oestrogen-dependent induction of cyclin D1 (Kirkegaard *et al.*, 2007; Planas-Silva *et al.*, 2001), as well as oestrogen-mediated cell survival (Weldon *et al.*, 2004). A NCOA3 splice variant found to be overexpressed was significantly more effective as a coactivator of oestrogen and progesterone compared to wild-type NCOA3 (Reiter *et al.*, 2001). Another study suggests that NCOA3 has a greater impact on ER activity than other coactivators, with suppression of the NCAO3 protein damaging ERα target gene regulation more than inhibition of NCOA1 (Shao *et al.*, 2004).

While NCOA3 appears to play an important role in hormone sensitive breast cancers, evidence is accumulating to support its role in hormone-insensitive breast cancers through its involvement in several signalling pathways. NCOA3 has been found to interact with E2F1, resulting in the transactivation of cyclin E, Cdk2, cyclin A, cdc25A as well as E2F1 itself (Louie *et al.*, 2006; Louie *et al.*, 2004), all of which are genes that are involved in cell proliferation. E2F1 and cyclin E have also been associated with a poor prognosis in breast cancer (Keyomarsi *et al.*, 1994; Loden *et al.*, 2002). The later study by Louie *et al.*, uncovered evidence that NCOA3 directly controls the expression of genes responsible for DNA replication, is important for successful G1-S progression of both normal and malignant human cells, and has the ability to transform normal human breast epithelial cells through its relationship with E2Fs (Louie *et al.*, 2006). Another study focussing on hormonal-insensitive breast cancers, found that NCOA3 mediates insulin-like growth factor cell proliferation, signalling, and cell survival (Oh *et al.*, 2004; Yan *et al.*, 2006). Interestingly, Yan *et al.* found that NCOA3 requires activator protein-1 (AP-1) when recruited to the promoter of IGF-I (Yan *et al.*, 2006). Recently, NCOA3 has also been implicated in pre-neoplastic changes in the mammary epithelium (Avivar *et al.*, 2006). In this study, transgenic mice overexpressing NCOA3 were found to develop mammary hyperplasia at the onset of puberty.

3.1.7 Retinoic Acid Receptor Responder 3

Mapped to chromosome 11q23, Retinoic Acid Receptor Responder 3 (RARRES3), also known as Retinoid-inducible gene (RIG1) and tazarotene-induced gene 3 (TIG3), can be induced by a synthetic retinoid tazarotene used clinically for the treatment of psoriasis (Duvic *et al.*, 2003). RARRES3 has 52% homology with the tumour suppressor H-rev107, a gene whose over-expression leads to resistance to transformation by activated H-ras oncogenes (DiSepio *et al.*, 1998). Similarly, RARRES3 promotes cell growth suppression and differentiation (DiSepio *et al.*, 1998; Sturniolo *et al.*, 2003). The expression of RARRES3 is reduced in many cancers such as Wilm's tumours (Zirn *et al.*, 2006), skin cancers (Duvic *et al.*, 2000) and ovarian cancers (Lotz *et al.*, 2005), and this low expression appears to correlate with well-differentiated tissues (Duvic *et al.*, 2003; Jiang *et al.*, 2005b; Shyu *et al.*, 2003). Recently however, overexpression of RARRES3 mRNA has been found in 44 out of 47 breast cancers (Shyu *et al.*, 2005) and in 5 nasopharyngeal tumourigenic cell lines (Kwong *et al.*, 2005). In the case of breast cancer, a correlation between RARRES3 expression and breast cancers lacking ER/PgR was found, and cell lines treated with

oestrogen reduced RARRES3 mRNA expression (Shyu *et al.*, 2005). Another study found amplification at11q12-q13 in breast tumours, the region that RARRES3 is mapped to (Nessling *et al.*, 2005). It has been reported that RARRES3 exerts its tumour suppressor effects by suppressing Ras activation, thereby inhibiting the MAP-kinase pathway (Tsai *et al.*, 2006). The same group also confirmed that RARRES3 possesses pro-apoptotic properties, which are executed via the activation of caspases-2 and -3 (Tsai *et al.*, 2006).

3.1.8 Retinoblastoma Binding Protein 4

Retinoblastoma Binding Protein 4 (RBBP4), mapped to chromosome 1p35.1, encodes for the retinoblastoma binding protein (Qian *et al.*, 1993), which is a component of several distinct nucleosome modifying complexes including: the nuclear histone deactylases (HDACs) (Hassig *et al.*, 1997), chromatin assembly factor 1 (CAF-1)(Kaufman *et al.*, 1997), and Hat1, a type B histone acetylase (Zhang *et al.*, 1997). Increased expression of RBBP4 has been found in thyroid (Pacifico *et al.*, 2007), hepatocellular (Song *et al.*, 2004), and non-small cell lung cancers (Fukuoka *et al.*, 2004), whereas, down-regulation was observed in mucoepidermoid carcinoma (Leivo *et al.*, 2005). The function of RBBP4 in carcinomas has not been fully elucidated, however, it seems that at least in thyroid cancers, RBBP4 is regulated by NF–kB and it supports proliferation (Pacifico *et al.*, 2007). In cervical cancer, RBBP4 is a key mediator in controlling the transforming activity of the human papillomavirus (HPV) (Kong *et al.*, 2007).

3.1.9 Transforming Growth Factor β Induced

The Transforming Growth Factor-β Induced (TGFβI) gene is located on chromosome 5q31 and encodes an extracellular matrix protein that can be induced by transforming growth factor β1 in many cell types, including mammary epithelial cells, human melanoma cells, keratinocytes, and lung fibroblasts (Skonier *et al.*, 1994). TGFβI appears to be involved in cell growth and differentiation (Skonier *et al.*, 1994) and has been found to bind *in vitro* to many matrix components including fibronectin, laminin, and several collagen types (Kim *et al.*, 2002). Mutations in TGFβI have been linked to numerous autosomal corneal dystrophies (Munier *et al.*, 1997), a disease caused by the build up of TGFβI-containing protein deposits in the corneal matrix (Clout & Hohenester, 2003). Functional analysis of TGFβI has shown that it is secreted into the extracellular matrix (ECM) and that it may act as an extracellular attachment protein, thereby controlling cell attachment and migration (LeBaron *et al.*, 1995).

More recently, TGFβI has been found to support adhesion, migration and proliferation of renal tubular epithelia cells and vascular smooth muscle cells through interaction with α3β1 integrin (Park *et al.*, 2004). The increased expression of TGFβI has been found in several cancers including, squamous cell carcinoma (Hu *et al.*, 2001), oesophageal (Hourihan *et al.*, 2003), pancreatic (Schneider *et al.*, 2002), colorectal (Buckhaults *et al.*, 2001), as well as in ovarian endometriosis (Arimoto *et al.*, 2003). Conversely, one study found a decreased TGFβI expression in an ER-positive ovarian cancer cell line (Walker *et al.*, 2007). The precise role of TGFβI in human cancers is not yet known. Since TGFβI is believed to be involved in cell attachment to the ECM, it is possible that any elevated levels of TGFβI may control cell adhesion or invasion of cancer cells in cancer progression.

3.1.10 Target gene summary

On the basis of the differential expression identified in the microarray study, and the reported function of the proteins encoded by the 9 genes (including cell cycle, differentiation, DNA repair, and cell adhesion) the expression of the nine selected genes was examined in breast cell lines, normal organoids from reductions mammoplasties, and a cohort of sporadic breast cancers from women stratified by age.

The results are presented as:

Gene expression

i. Optimisation
ii. Data from *in vitro* cell lines
iii. Data from *in vivo* tissues

Protein expression

i. Western blotting in cell lines and tissues
ii. Immunohistochemistry in tissues
iii. Comparison between mRNA and protein expression for NCOA3

3.2 Gene expression optimisation

3.2.1 RNA extraction from FFPE tissue

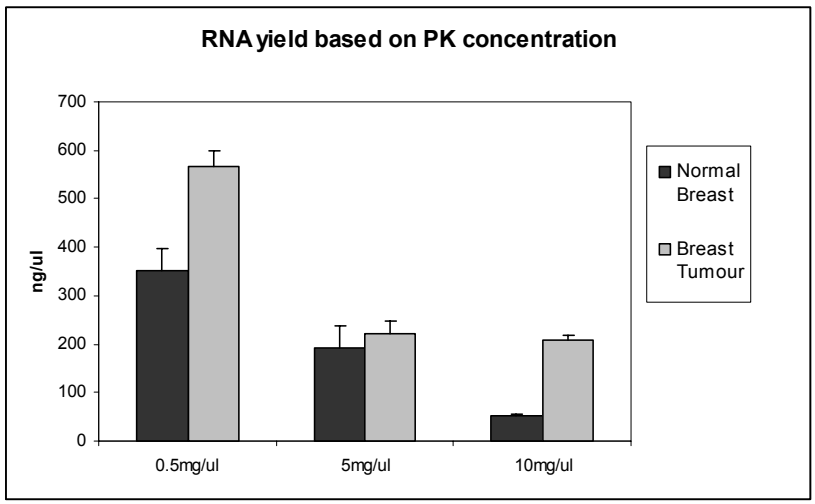
The preparation of DNA from formalin fixed, paraffin-embedded (FFPE) tissue is a relatively straight forward technique. However, the preparation of RNA from FFPE is less simple, as formalin degrades RNA, crosslinks proteins that shear RNA, and covalently modifies RNA by addition of monomethylol groups (Masuda *et al.*, 1999).

To ensure that mRNA could be effectively isolated from FFPE samples it was necessary to first optimise the conditions for tissue digestion using Proteinase K (PK). To do this, one normal breast sample and one tumour sample, both with ample tissue available, were used. Ten 4 μm tissue sections were dewaxed, rehydrated and incubated at different concentrations of PK (0.5mg/ml, 5mg/ml, and 10mg/ml), at varying temperatures (37ºC and 50ºC) and different incubation periods (12 hours, 48 hours, and 60 hours). Following incubation, RNA was isolated using the Phenol:chloroform method outlined in section 2.2.3.

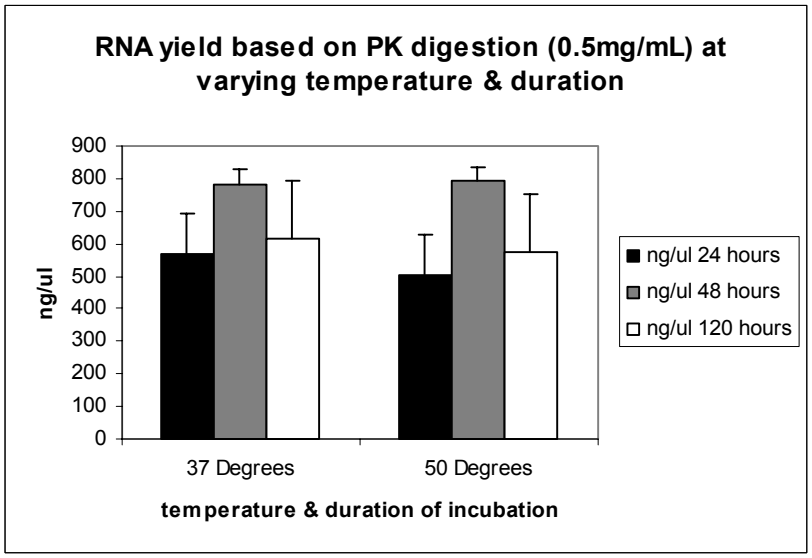
RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm was used to assess the RNA purity, with a ratio between 1.8 and 2.0 being desireable. A PK concentration of 0.5mg/ml was most successful at digesting the tissue to produce higher yields of RNA than the more concentrated solutions (see Figure 3.1A). While incubating the samples for 48 hours at 37ºC was the optimal incubation period for higher RNA yields (see Figure 3.1 B), the RNA was less pure than RNA that was from 120 hours incubation (see Figure 3.1C). However, this difference in purity was small and the purity of 1.81 (0.5mg/ml at 37 ºC for 48 hours) was well within the ideal range of 1.8 to 2.0 for RNA. Therefore for all subsequent RNA preparations from FFPE tissue, the PK digestions were carried out at 0.5mg/ml PK at 37 ºC for 48 hours, followed by the Phenol:chloroform step.

Figure 3.1 Optimisation of RNA extraction from FFPE tissue using PK

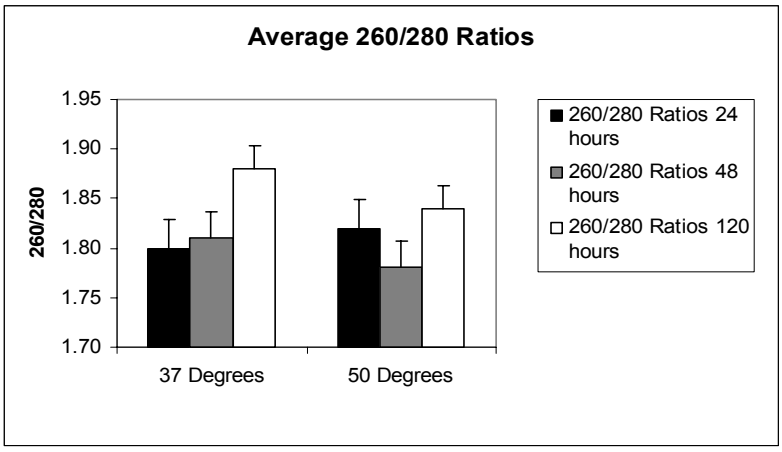
A.



B.



C.

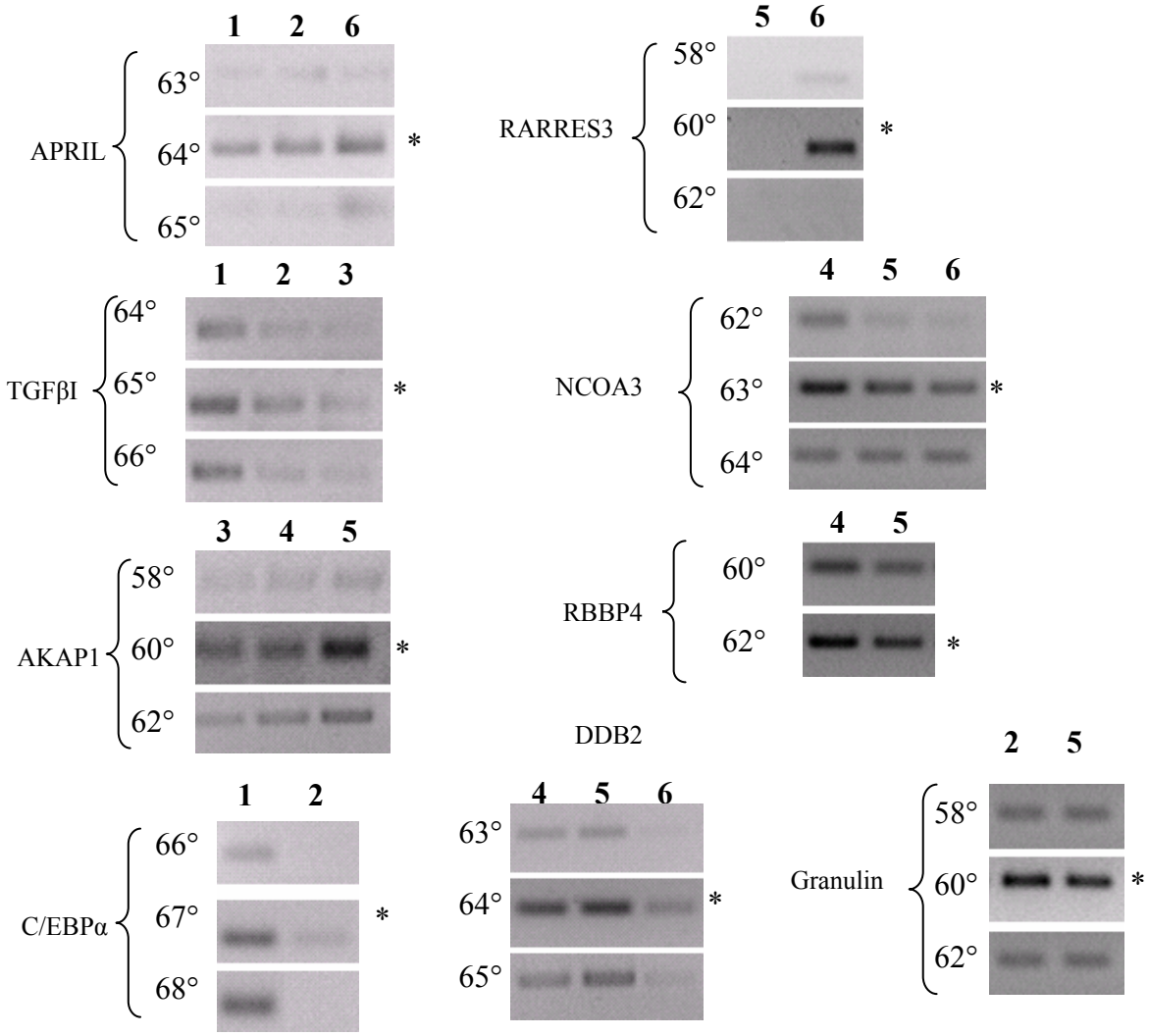


A: RNA yield based on Proteinase K concentration during incubation; B: RNA yield based on the temperature and duration of incubation (normal breast tissue was used); C: RNA purity (normal breast tissue was used)

3.2.2 Primer Optimisation

Primer pairs were optimised for all nine target genes for the most appropriate annealing temperature using manual RT-PCR. Briefly, the optimum annealing temperature was selected based on the highest yield of PCR product at the expected size with no evidence of accompanying primer dimer formation or non-specific products. For example, for NCOA3, 63º gave good amplification with no primer dimers or non-specific products (see Figure 3.2). Lower yields were observed at 62ºC and 64ºC. Results for optimisation of all nine target gene primers are displayed in Figure 3.2 and summarised in Table 3.2.

Figure 3.2 Optimisation of target gene primer pairs



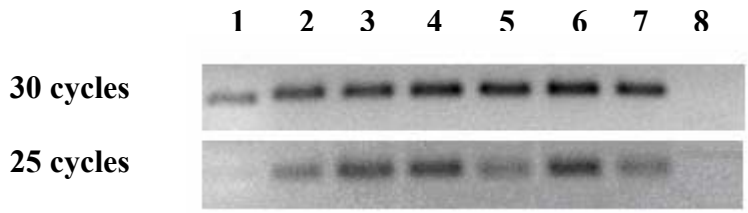
Agarose gel analysis of RT-PCR products from breast cell lines that were positive for amplification of each target gene

Cell Lines used: 1, HBL-100; 2; MDA-MB-231; 3, MCF-7; 4, ZR-75-1; 5, T47-D; 6, MDA-MB-468.

* optimised annealing temperature chosen for subsequent PCR reactions.

Primer pairs were also optimised for the number of PCR cycles to allow comparison of relative levels of expression. Primer pairs were initially run for 30 cycles. If the yield of PCR products was too low to allow comparison, the cycles were increased to 35 cycles. However, if differences in PCR product yields were indistinguishable, cycles were reduced to 25 cycles. In practice, all primer pairs for the 9 target genes worked well at 30 cycles, except for granulin which appeared to be saturated when run at 30 cycles (see Figure 3.3). Optimised cycle numbers for each target gene are summarised in Table 3.2.

Figure 3.3 Granulin RT-PCR comparing 25 and 30 cycles



Lanes: 1, 100bp DNA ladder ; 2, HBL-100; 3, MDA-MB-231; 4, MCF-7; 5, ZR-75-1; 6, T-47-D; 7, MDA-MB-468; 8 , Water Blank

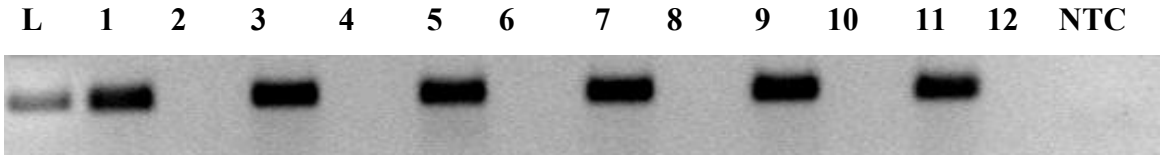
Table 3.2 Optimisation of annealing temperature and number of cycles for PCR

Primer Pair	Annealing Temperature (C°)	No. of cycles
NCOA3	63	30
AKAP1	60	30
DDB2	64	30
APRIL	64	30
C/EBPα	67	30
TGFβI	65	30
RARRES3	60	30
Granulin	60	25
RBBP4	62	30
HPRT1	57	30
PBGD	60	30
TFRC	57	30

3.2.3 Housekeeping gene analysis

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a widely used housekeeping gene, was used to ensure comparability between yields of template cDNA for RT-PCR (see Figure 3.4). First strand cDNA was synthesised from 1 μg of RNA template for all cell lines, to yield approximately equivalent amounts of cDNA. Each PCR reaction was run for 30 cycles. However, small variations were seen due to experimental differences in RT efficiency and slight pipetting errors. To control for the RT step and also examine the possibility of genomic DNA carry over during RNA extraction, negative reverse transcription (-RT) reactions were carried out. These contained RNA and all reverse transcription reagents except AMV-RT. They were analysed for GAPDH; the absence of GAPDH PCR product indicated no genomic DNA contamination (see Figure 3.4).

Figure 3.4 Housekeeping gene (GAPDH) analysis in 6 cell lines



L 100bp DNA ladder; 1, HBL-100; 2, HBL-100 –RT; 3, MDA-MB-231; 4, MDA-MB-231–RT; 5, MCF-7; 6, MCF-7–RT; 7, ZR-75-1; 8, ZR-75-1 RT; 9, T47-D; 10, T47-D-RT; 11, MDA-MB-468; 12, MDA-MB-468-RT. NTC No template control.

3.2.4 Real time quantitative RT-PCR

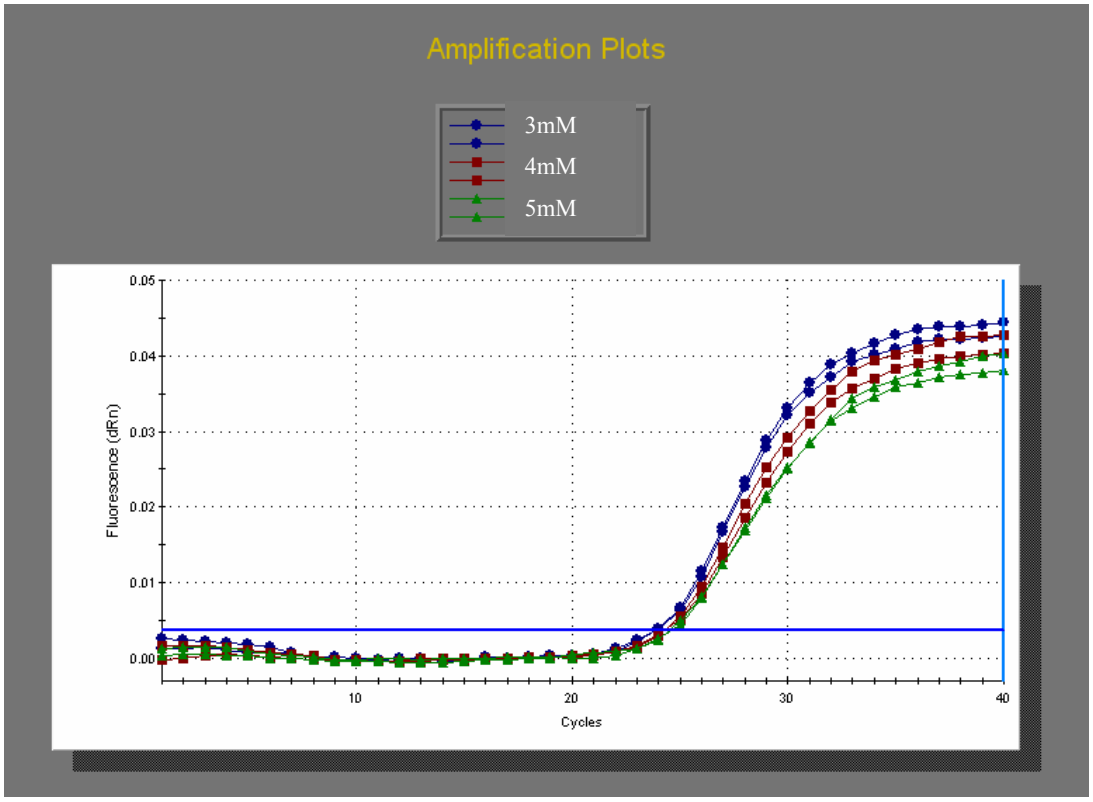
3.2.4.1 Primer optimisation

All primer pairs were tested to determine the optimum concentration of $MgCl_2$ that gave the lowest cycle threshold. The PCR master mix (Invitrogen ™) had a $MgCl_2$ concentration of 3 mM. To verify whether each primer pair required greater than 3 mM of $MgCl_2$, final concentrations of 3mM, 4 mM and 5 mM $MgCl_2$ were first used in the optimisation experiments. All optimisation experiments for each target primer set were carried out on a selected cell lines that expressed the gene (see Table 3.3), and all experiments were carried out in duplicate. The results showed that a $MgCl_2$ concentration of 3mM was optimal for each primer pair. For example, the optimum $MgCl_2$ for AKAP1 was 3mM giving the lowest cycle threshold (Ct = 23.90) compared to concentrations of 4mM (Ct = 24.33) and 5 mM (Ct = 24.59) (see Figure 3.5). Raw Ct values for each optimisation experiment are shown in Table 3.4, and a summary of $MgCl_2$ concentrations used in all subsequent experiments is shown in Table 3.5

Table 3.3 Cell lines used for optimisation of real-time qRT-PCR experiments

Target gene	Positive cell line
AKAP1	T47-D
APRIL	MDA-MB-231
C/EBP alpha	HBL-100
DDB2	T47-D
Granulin	MDA-MB-231
NCOA3	ZR-75-1
RARRES3	MDA-MB-468
RBBP4	ZR-75-1
TGFβI	HBL-100
HPRT	HBL-100
PBGD	HBL-100
TFRC	HBL-100

Figure 3.5 Optimisation of the AKAP1 primer pair for $MgCl_2$ concentration



RT-PCR of AKAP1 using the T47-D cell line. All reactions were carried out in duplicate.

Table 3.4 Raw Ct values for primer pair optimisation of $MgCl_2$

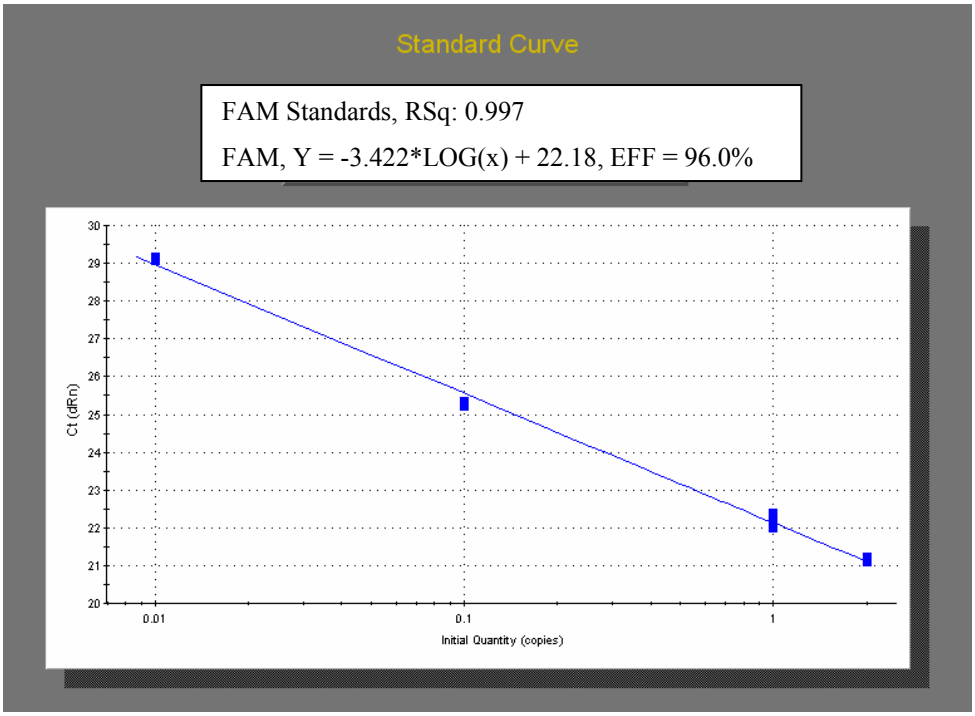
Primers	Concentration	1	2	Mean
NCOA3	3mM	22.10	22.04	22.07
	4mM	23.06	23.12	23.09
	5mM	24.08	23.98	24.03
AKAP1	3mM	23.91	23.89	23.90
	4mM	24.33	24.33	24.33
	5mM	24.53	24.66	24.60
DDB2	3mM	25.00	29.64	27.32
	4mM	25.09	24.99	25.04
	5mM	28.15	25.19	26.67
APRIL	3mM	20.25	20.11	20.18
	4mM	20.37	20.42	20.40
	5mM	20.62	20.61	20.62
C/EBPα	3mM	29.30	29.19	29.25
	4mM	29.77	29.87	29.82
	5mM	30.29	30.37	30.33
TGFβI	3mM	32.63	32.13	32.38
	4mM	33.19	33.01	33.10
	5mM	32.91	32.90	32.91
RARRES3	3mM	23.03	23.07	23.05
	4mM	25.85	25.94	25.90
	5mM	26.03	26.10	26.07
Granulin	3mM	20.21	20.15	20.18
	4mM	20.04	20.17	20.11
	5mM	20.36	20.34	20.35
RBBP4	3mM	22.51	22.61	22.56
	4mM	23.15	23.23	23.19
	5mM	23.01	23.51	23.26
HPRT	3mM	25.02	25.10	25.06
	4mM	25.48	25.77	25.63
	5mM	25.80	26.07	25.94
TFRC	3mM	24.58	25.67	24.63
	4mM	25.04	25.12	25.08
	5mM	25.11	25.09	25.10
PGBD	3mM	19.74	19.87	19.80
	4mM	20.05	20.11	20.08
	5mM	20.08	20.09	20.09

All reactions were run in duplicate (1 and 2).

3.2.4.2 Standard Curve

For each primer pair, standard curves were constructed to calculate the PCR efficiency. To do this, cDNA from selected cell lines expressing the target gene (see Table 3.3) was serial diluted and amplified, to produce standard curves expressing a linear relationship between template quantity and target gene expression. PCR efficiency was calculated from the gradient of the standard curve according to Equation 2.2.8.3.1. For example, RBBP4 had a PCR efficiency of 96.0% calculated from the standard curve (see Figure 3.6). The PCR efficiency for each primer pair are summarised in Table 3.5.

Figure 3.6 Generation of standard curve for RBBP4 RT–qPCR



RBBP4 amplification in ZR-75-1 (2µL, 1 µL, 0.1 µL, and 0.01 µL). Each reaction was carried out in triplicate.

Table 3.5 Summary of optimum $MgCl_2$ concentrations and PCR efficiency for primer pairs

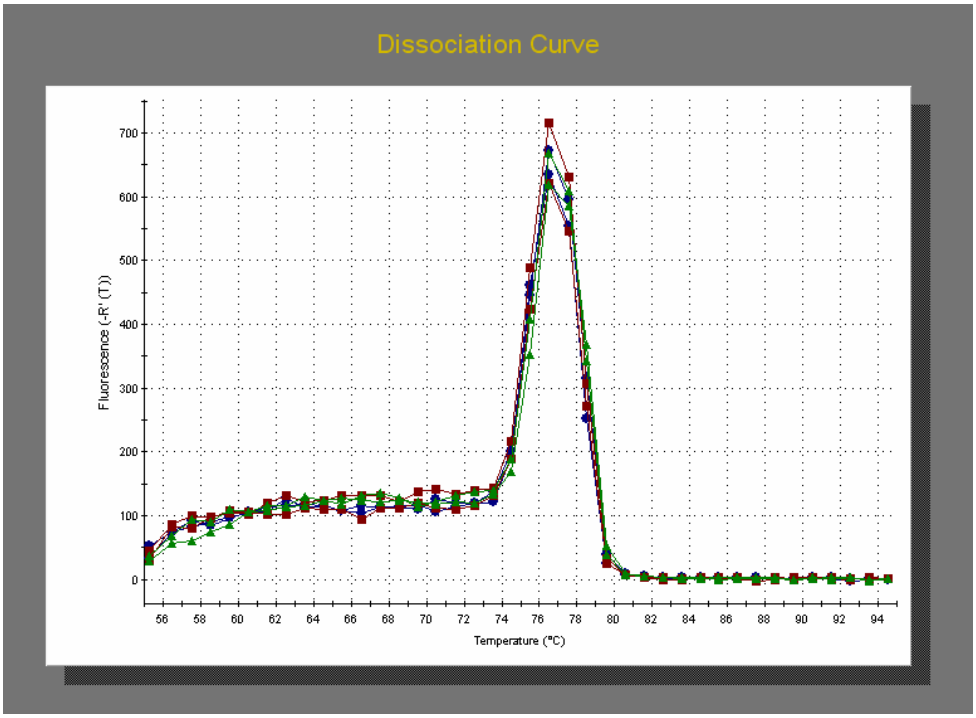
Primer Pair	Optimum $MgCl_2$ concentration (mM)	PCR Efficiency (%)
AKAP1	3	82.7
APRIL	3	96.0
C/EBPα	3	98.4
DDB2	3	87.2
Granulin	3	109.0
NCOA3	3	104.8
RARRES3	3	101.1
RBBP4	3	96.0
TGFβI	3	100.2
PBGD	3	100.7
TFRC	3	105.8
HPRT	3	99.2

Despite extensive attempts at refining the optimisation the efficiency values for DDB2 and AKAP1 were below the recommended 90% (87.2% and 82.7% respectively). However, it was agreed that RT-qPCR experiments should proceed since the relative expression level of both targets was calculated using the efficiency of both the target gene and reference gene. The results should therefore give a reasonable representation of target gene expression in each sample.

3.2.4.3 Dissociation curves

In order to ensure that any increase in fluorescence was due to cDNA amplification for the gene of interest and not attributable to non-specific products or primer dimer artefacts, dissociation curves were plotted for all PCR products. Dissociation curves for all genes analysed were shown to be specific with only a single peak at the melting temperature of the PCR product. Figure 3.7 shows an example of the dissociation curve for APRIL.

Figure 3.7 Dissociation curve for APRIL



The careful optimisation for annealing temperatures and $MgCl_2$ concentrations, as well as the verification of a single PCR product for each primer set, ensured that all subsequent RT-qPCR experiments in this project were performed at the optimal conditions that allowed accurate detection of the expression of each target gene in all cell lines and tissues. In each assay a standard curve was carried out to control for inter assay variability.
The full data for standard curves and dissociation curves are given in Appendix II.

3.2.4.4 Housekeeping gene selection

Real-time qPCR is a sensitive and accurate technique for measuring target mRNA expression. A key step of this technique is normalisation of the results to compensate for differences in the purity and concentration of the samples. The most commonly used normalisers in real-time PCR are endogenous reference genes or housekeeping genes. Ideally, housekeeping genes should be ubiquitously expressed at similar levels in all samples and experimental conditions. Unfortunately, many commonly used housekeeping genes such as GAPDH (Barber *et al.*, 2005; Tricarico *et al.*, 2002), and beta-actin (Tricarico *et al.*, 2002; Vandesompele *et al.*, 2002) vary across tissue types and under experimental conditions makes choosing an appropriate housekeeping gene challenging. Following a review of other

published studies, 3 housekeeping genes were selected for validation (see Table 3.6): hypoxanthine phosphoribosyltransferase (HPRT1), Transferrin receptor (TFRC), and porphobilogen deaminase (PBGD).

Table 3.6 Performance of HPRT, TFRC and PGBD as housekeeping genes in previous studies

Findings	Reference
In breast, HPRT, PGBD, and TFRC were the highest ranked genes following analysis of the expression patterns of 13 frequently used housekeeping genes in 80 normal and tumor samples from colorectal, breast, prostate, skin, and bladder tissues with real-time quantitative RT-PCR.	(de Kok *et al.*, 2005)
Abundance of HPRT and PGBD found to be relatively similar across 13 different human tissues	(Vandesompele *et al.*, 2002)
PGBD showed a relatively constant expression over a variety of cell lines and tumours	(Janssens *et al.*, 2004)

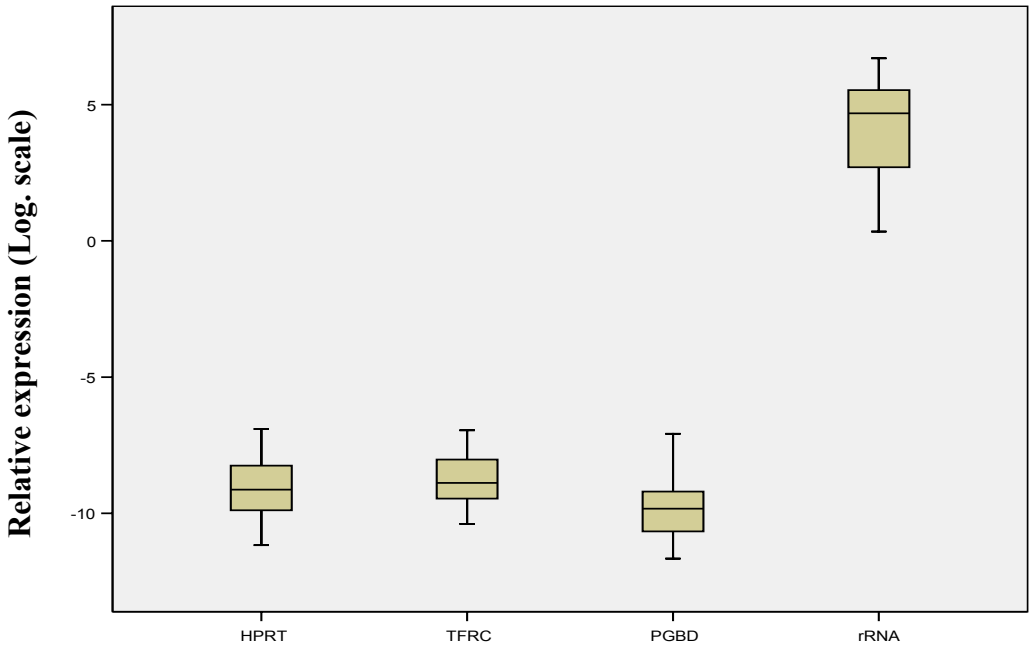
In order to select one of these housekeeping genes for all subsequent target gene analyses, it was impotant to ensure the housekeeping gene was expressed at constant levels in all samples. Therefore the expression of all 3 genes was first measured in cell lines, organoids, and all tumour tissues (59 samples in total). 1μg of RNA was used for cDNA synthesis and RT-qPCR (see section 2.2.4 for conditions). All reactions were carried out in triplicate. Variation in the expression levels was observed in all 3 housekeeping genes across 59 samples (see Figure 3.8). PGBD had the greatest variation, followed by HPRT1. TFRC had the least variation. However, an F-test showed no significant difference between the variability in HPRT1, TFRC, and PGBD (F=0.75, 0.59, and 0.79). Overall, the level of housekeeping gene detected was higher in the fresh cell lines, organoids, and snap frozen tissue compared to the FFPE tissue (see Figure 3.9).

Towards the end of the project the housekeeping gene selection was reevaluated. 18S ribosomal RNA - a stable internal control with low expression variance was tested as a potential final control, since it has been reported to be effective in other studies (Schmittgen & Zakrajsek, 2000; Tsuji *et al.*, 2002; Zhang *et al.*, 2007). Due to limited sample quantities, 18S could only be tested in 36 out of 59 samples. As expected, 18S was expressed at higher

levels than HPRT1, TFRC, and PGBD (see Figure 3.8 and Figure 3.9). However as for the 3 housekeeping genes, 18S also showed variation in its expression among all samples (see Figure 3.8). When the expression of 18S was reduced by a factor of 10^{12}, it displayed expression levels more similar to those of the 3 housekeeping genes (see Figure 3.10). However, despite this, the wide variability in expression across all samples remained.

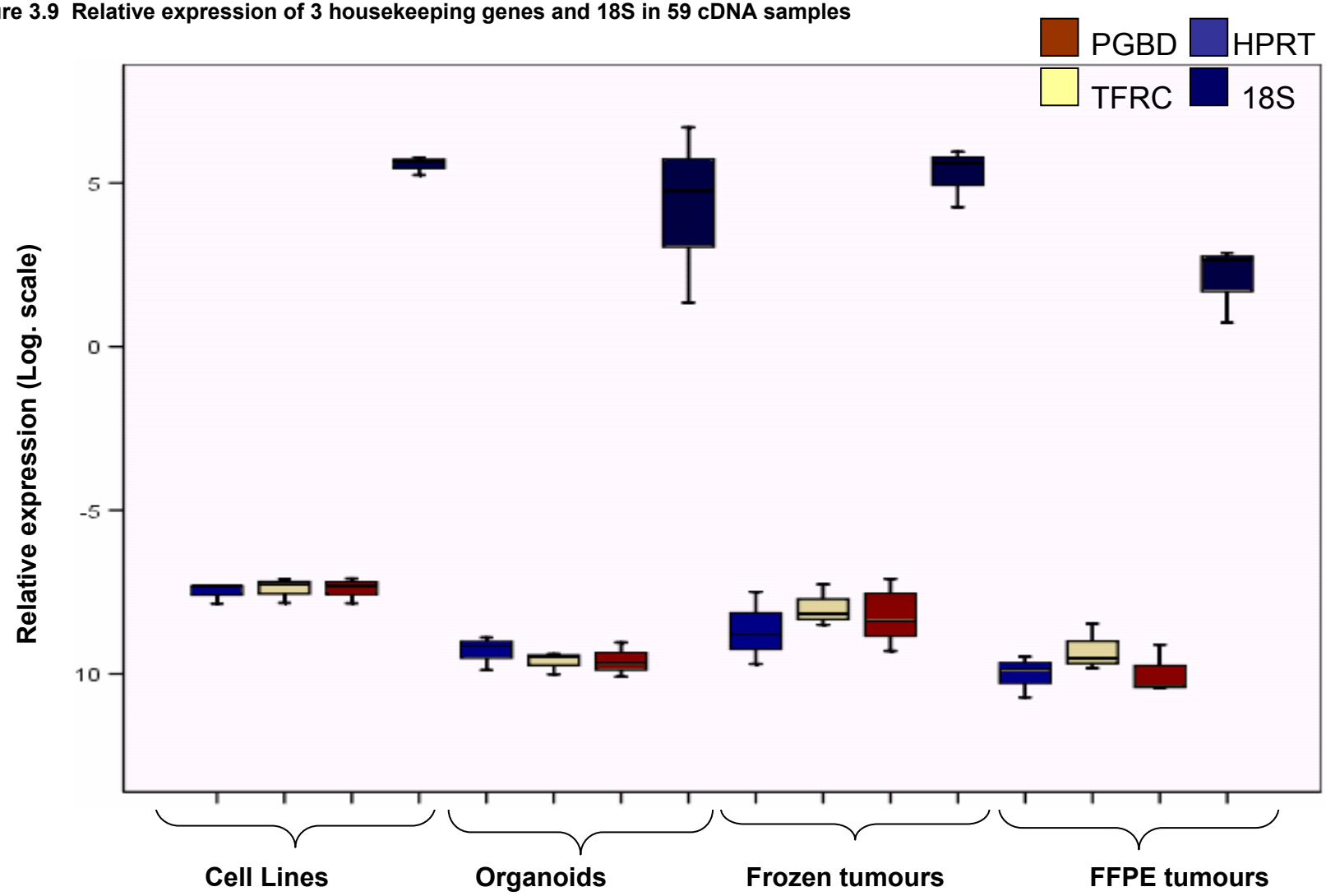
Currently, the best option in normalisation is to measure the expression of multiple housekeeping genes and normalise using their mean expression. Since HPRT1, TFRC, and PGBD displayed similar levels of expression with least variation, all subsequent target gene expression analyses were normalised using the average of the expression of HPRT, TFRC, and PGBD, since this would provide a more accurate normaliser than using a single housekeeping gene.

Figure 3.8 Variation in housekeeping gene expression among 59 cDNA samples



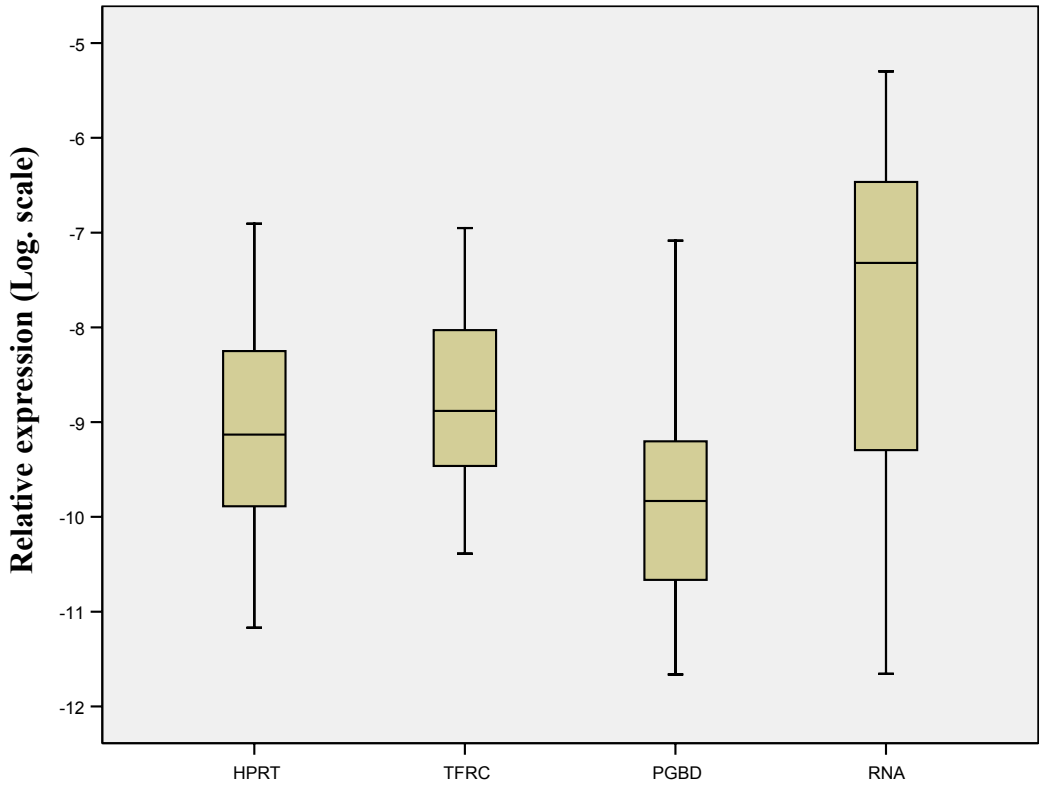
1µg amount of RNA was used for cDNA synthesis and RT-qPCR. 59 samples were examined in total. Box plots represent the relative expression of each housekeeping gene in all 59 samples. Error bars represent the standard deviation.

Figure 3.9 Relative expression of 3 housekeeping genes and 18S in 59 cDNA samples



1µg amount of RNA was used for cDNA synthesis and RT-qPCR. 59 samples were examined in total. Box plots represent the relative expression of each housekeeping gene in all 59 samples across all sample types. Error bars represent the standard deviation.

Figure 3.10 Effect of reduction of 18S by a factor of 10^{12}



1µg amount of RNA was used for cDNA synthesis and RT-qPCR. 59 samples were examined in total. Box plots represent the relative expression of each housekeeping gene in all 59 samples. 18S expression is reduced by a factor of 12. Error bars represent the standard deviation.

3.3 Target gene expression in breast cell lines

Six different breast cell lines (HBL-100, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T47-D) were chosen for analysis as the first line of investigation into the validation of the nine selected target genes. One microgram of total RNA was extracted from the six breast cell lines and converted to cDNA using reverse transcriptase as outlined in sections 2.2.3.1 and 2.2.7.1. The cDNA was then examined for the expression of the nine target genes using manual PCR, followed by a real-time quantitative PCR (see section 2.2.7-8).
For real-time quantitative PCR, the relative amount of each transcript was determined using the PCR efficiency and cycle number at which the fluorescence crossed a cycle threshold (Ct) (Pfaffl, 2001):

$$\text{Transcript quantity} = 1/(\text{Efficiency of PCR})^{\text{Ct value}}$$

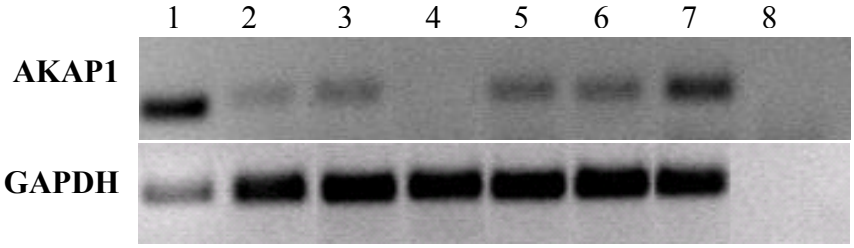
The final results were expressed as target gene expression relative to the average of the 3 housekeeping genes (relative expression), whereas in the microarray, expression data was represented as a fold change generated after normalising each target gene against either the organoids or HBL-100 cell line. All raw Ct values can be found in Appendix III.

3.3.1 AKAP1 expression

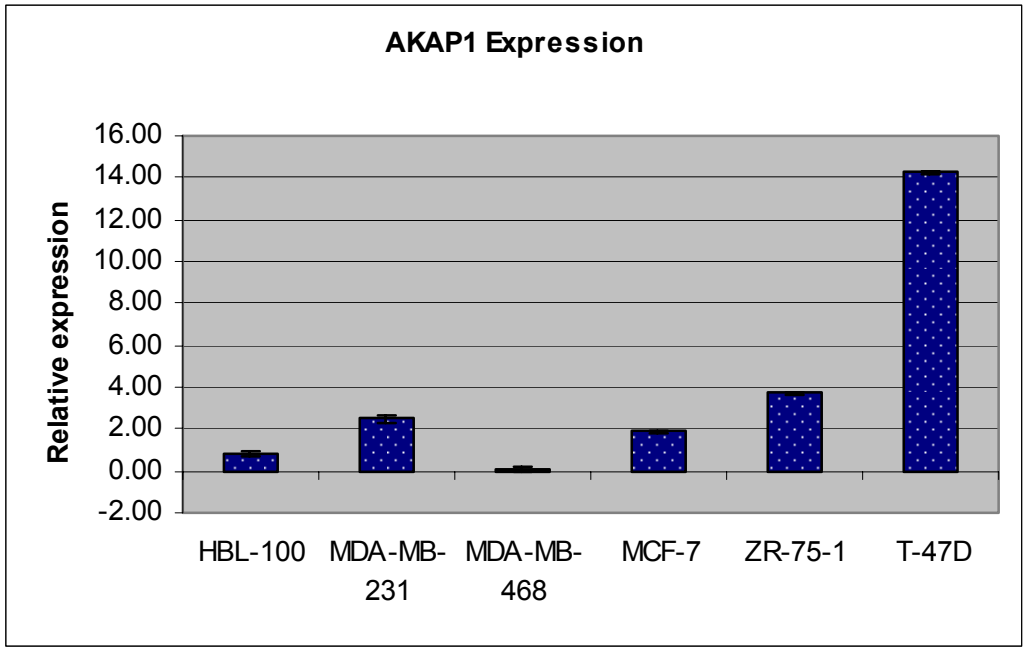
Manual RT-PCR showed a single product of the expected size (105 base pairs) in 5 of 6 cell lines (see Figure 3.11A). No expression was seen in the MDA-MB-468 cell line. The intensity was stronger in the tumourigenic cell lines, MDA-MB-231, MCF-7, ZR-75-1, and T47-D compared to the non-tumourigenic HBL-100.
Analysis of AKAP1 expression using real-time qPCR supported the pattern in expression observed by manual PCR (see Figure 3.11 B). MDA-MB-231, ZR-75-1, and T47-D each had relative expression greater then 2, with that of T47-D reaching 14.26.

Figure 3.11 AKAP1 expression in 6 cell lines

A.



B.



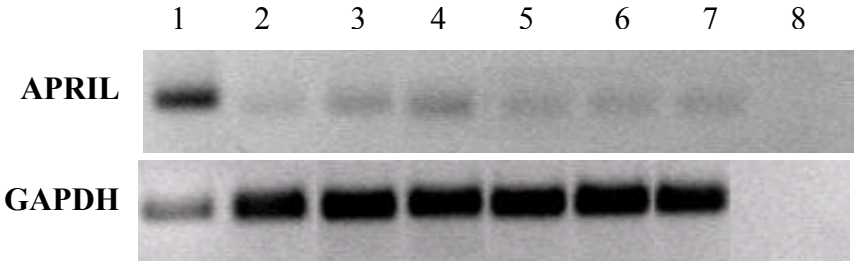
A: AKAP1 expression in 6 cell lines. The bottom gel picture shows GAPDH expression in the same cell lines. Lanes: 1, 100bp DNA ladder ; 2, HBL-100; 3, MDA-MB-231; 4, MDA-MB-468; 5, MCF-7; 6, ZR-75-1; 7, T-47-D; 8 , Water Blank. B: Relative AKAP1 expression in 6 cell lines using RT-qPCR. RT-qPCR experiments were carried out in triplicate (n=18). Error bars reflect standard error of the mean (SEM).

3.3.2 APRIL expression

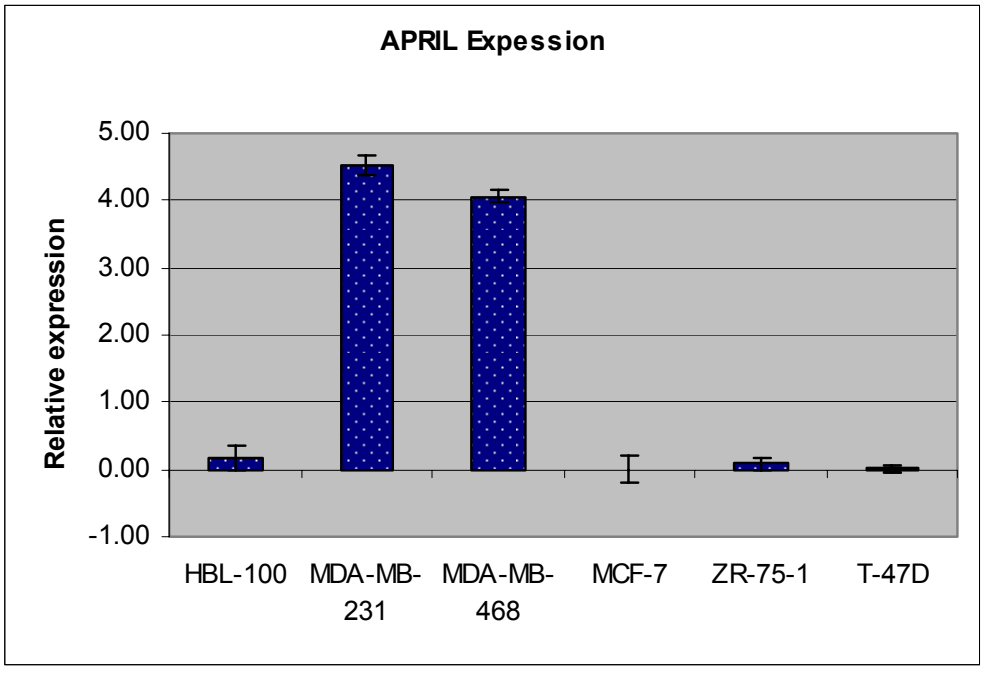
Manual PCR and real-time qPCR both showed APRIL expression was higher in MDA-MB-231, and MDA-MB-468 compared to the non-tumourigenic HBL-100 line (see Figure 3.12 A and B). The ER-positive breast cancer cell lines (MCF-7, ZR-75-1, and T47-D) all showed very low expression, similar to that of HBL-100.

Figure 3.12 APRIL expression in 6 cell lines

A.



B.



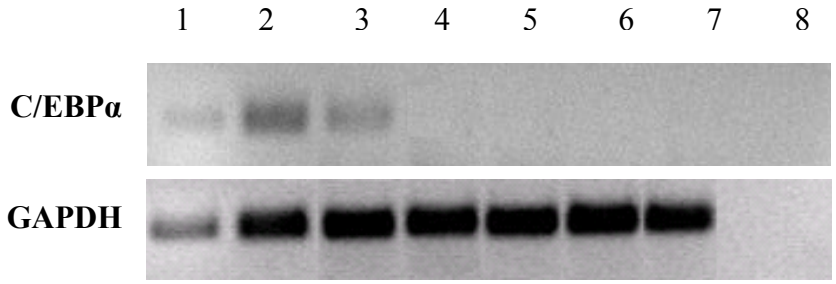
A**:** APRIL expression in 6 cell lines. The bottom gel picture shows GAPDH expression in the same cell lines. Lanes: 1, 100bp DNA ladder ; 2, HBL-100; 3, MDA-MB-231; 4, MDA-MB-468; 5, MCF-7; 6, ZR-75-1; 7, T-47-D; 8 , Water Blank. B: Relative APRIL expression in 6 cell lines using RT-qPCR. Rt-qPCR experiments were carried out in triplicate (n=18). Error bars reflect standard error of the mean (SEM).

3.3.3 C/EBP alpha Expression

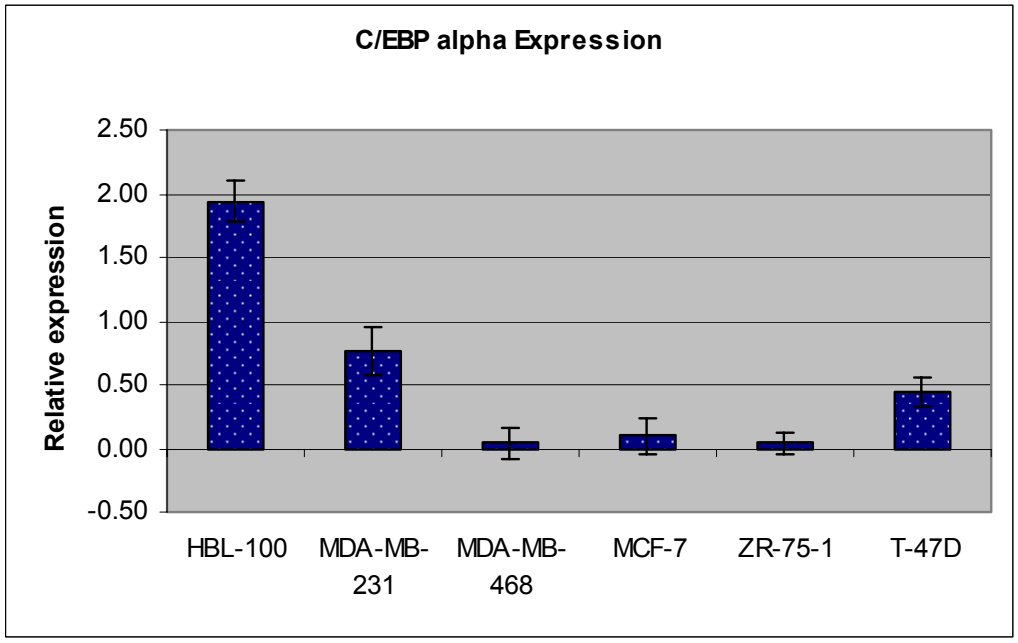
Manual RT-PCR showed a single product of the expected size, 103 base pairs in HBL-100 and MDA-MB-231 only (see Figure 3.13 A). However, expression was weaker in MDA-MB-231 compared to HBL-100. C/EBPα appeared absent in all other cell lines. The qPCR analysis supported these results, with expression patterns among the cell lines mirroring those observed in the manual PCR (see Figure 3.13 B). These results differ to those from the microarray, where C/EBPα was found to be up-regulated in the two tumours.

Figure 3.13 C/EBP alpha expression in cell lines

A.



B.



A: C/EBPα expression in 6 cell lines. The bottom gel picture shows GAPDH expression in the same cell lines. Lanes: 1, 100bp DNA ladder ; 2, HBL-100; 3, MDA-MB-231; 4, MDA-MB-468; 5, MCF-7; 6, ZR-75-1; 7, T-47-D; 8 , Water Blank. B: Relative C/EBPα expression in 6 cell lines using RT-qPCR. RT-qPCR experiments were carried out in triplicate (n=18). Error bars reflect standard error of the mean (SEM).

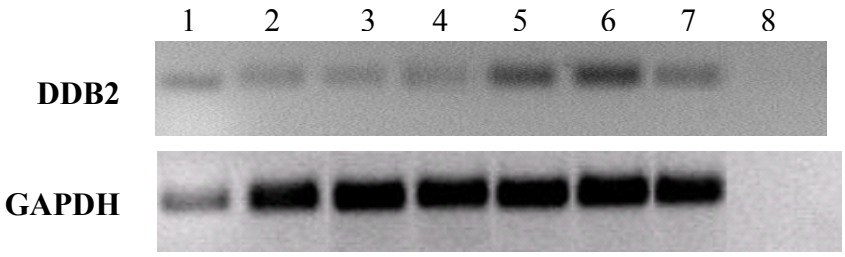
3.3.4 DDB2 expression

DDB2 expression was higher in ER-positive cell lines MCF-7, ZR-75-1, and T47-D compared to the non-tumourigenic HBL-100 cell line (see Figure 3.14 A). MDA-MB-231 and MDA-MB-468 had expression levels similar to HBL-100.

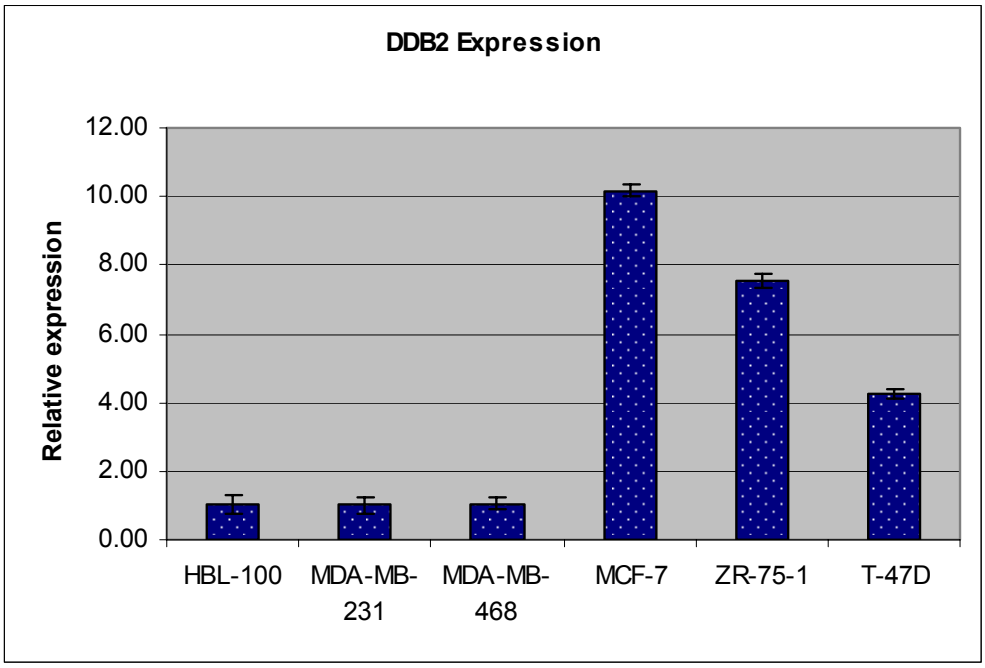
The qPCR supported the manual PCR analysis (see Figure 3.14 B). However, the relative expression of DDB2 in MCF-7 was higher than that of ZR-75-1, while in the manual PCR, ZR-75-1 appeared to have a slightly higher expression level. Since qPCR was carried out in triplicate and was highly reproducible, this is likely to show the most accurate data.

Figure 3.14 DDB2 Expression in cell lines

A.



B.



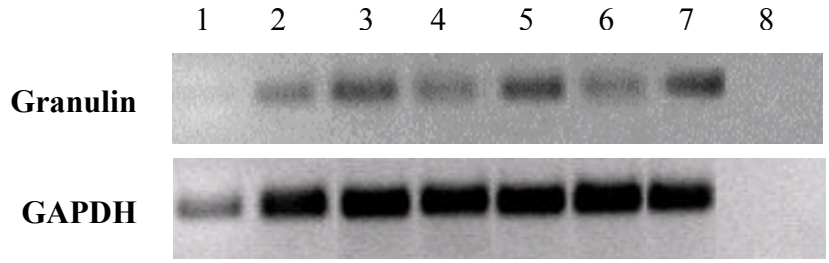
A: DDB2 expression in 6 cell lines. The bottom gel picture shows GAPDH expression in the same cell lines. Lanes: 1, 100bp DNA ladder ; 2, HBL-100; 3, MDA-MB-231; 4, MDA-MB-468; 5, MCF-7; 6, ZR-75-1; 7, T-47-D; 8 , Water Blank. B: Relative DDB2 expression in 6 cell lines using RT-qPCR. RT-qPCR experiments were carried out in triplicate (n=18). Error bars reflect standard error of the mean (SEM).

3.3.5 Granulin Expression

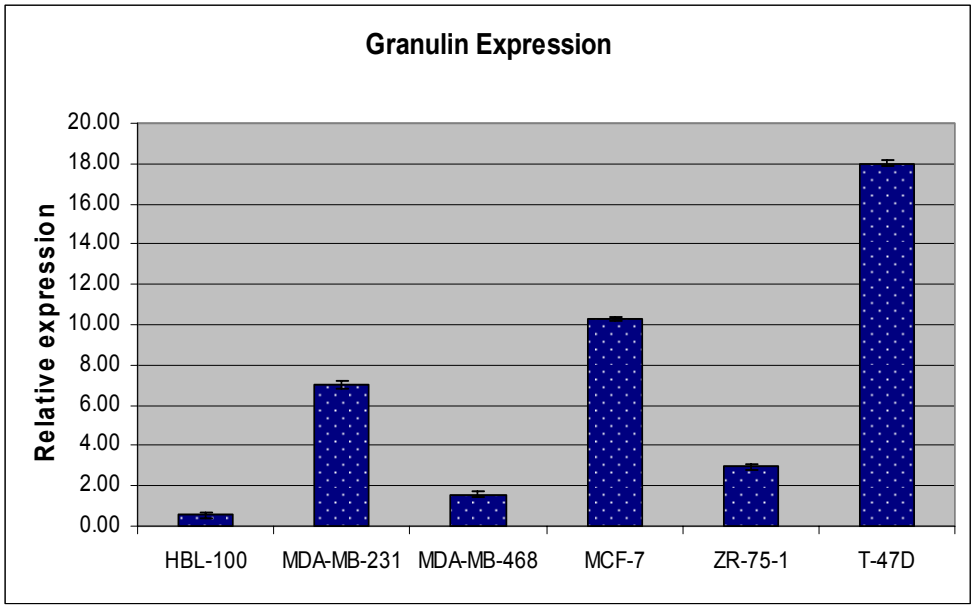
Manual RT-PCR showed a single product of the expected size, 99 base pairs, in all 6 cell lines (see Figure 3.15 A). MDA-MB-231, MCF-7, ZR-75-1, and T47-D showed higher granulin expression than MDA-MB-468 and HBL-100, which was in agreement with the data from the real-time qPCR (see Figure 3.15 B).

Figure 3.15 Granulin expression 6 cell lines

A.



B.



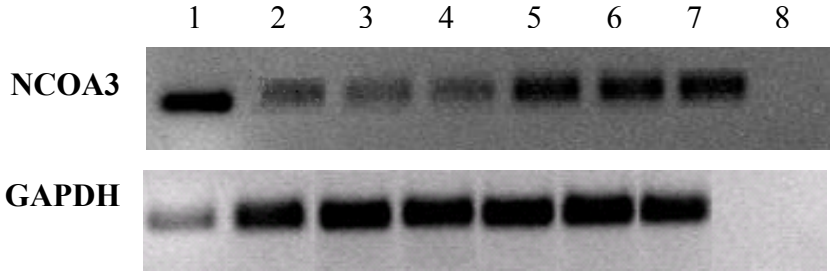
A: Granulin expression in 6 cell lines. The bottom gel picture shows GAPDH expression in the same cell lines. Lanes: 1, 100bp DNA ladder ; 2, HBL-100; 3, MDA-MB-231; 4, MDA-MB-468; 5, MCF-7; 6, ZR-75-1; 7, T-47-D; 8 , Water Blank. B: Relative DDB2 expression in 6 cell lines using RT-qPCR. RT-qPCR experiments were carried out in triplicate (n=18). Error bars reflect standard error of the mean (SEM).

3.3.6 NCOA3 Expression

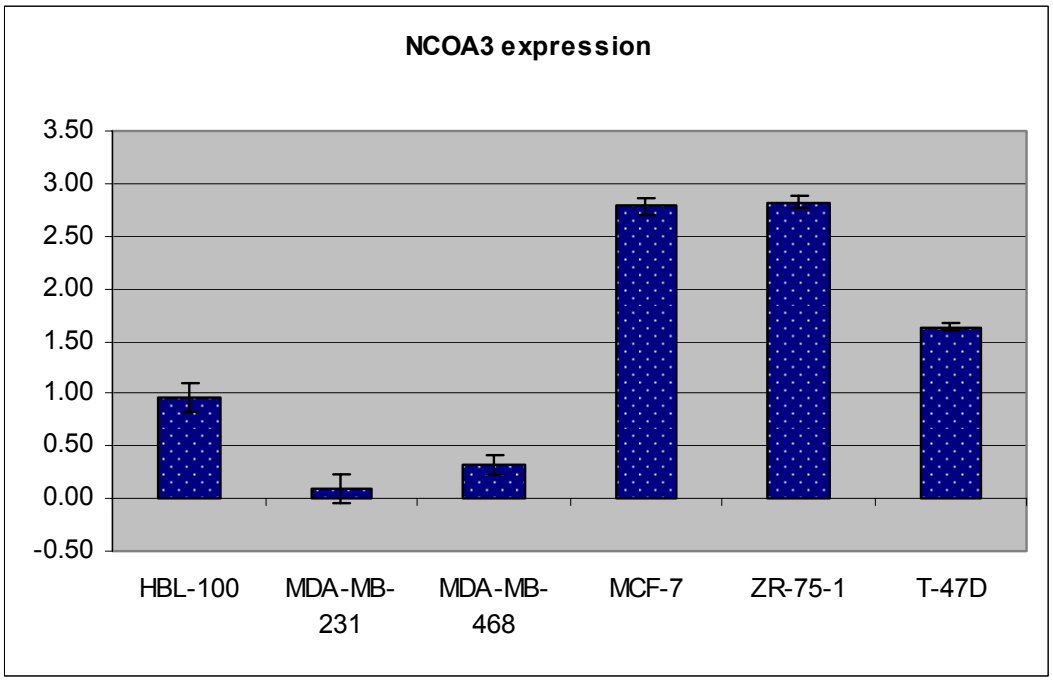
Both the manual PCR and real-time qPCR showed expression of NCOA3 to be higher in the ER-positive cell lines MCF-7, ZR-75-1, and T47-D, compared to all other cell lines (see Figure 3.16 A and B). While the expression of NCOA3 appeared strong in T47-D in the manual PCR, it appeared less intense in the qPCR. Expression was slightly lower in MDA-MB-231, and MDA-MB-468 compared to the non-tumourigenic HBL-100. This difference was more noticeable in the qPCR data.

Figure 3.16 NCOA3 Expression in cell lines

A.



B.



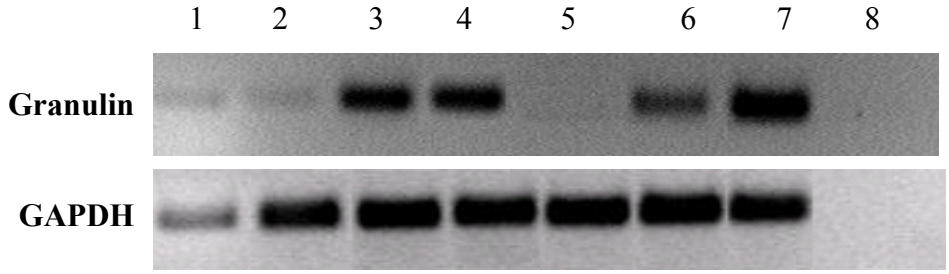
A. NCOA3 expression in 6 cell lines. The bottom gel picture shows GAPDH expression in the same cell lines. Lanes: 1, 100bp DNA ladder ; 2, HBL-100; 3, MDA-MB-231; 4, MDA-MB-468; 5, MCF-7; 6, ZR-75-1; 7, T-47-D; 8 , Water Blank. B: Relative NCOA3 expression in 6 cell lines using RT-qPCR. RT-qPCR experiments were carried out in triplicate (n=18). Error bars reflect standard error of the mean (SEM).

3.3.7 RBBP4 Expression

RBBP4 expression was higher in MDA-MB-231, MDA-MB-468, ZR-75-1, and T47-D compared to the non-tumourigenic HBL-100 line (Figure 3.17A and B). MCF-7 showed no RBBP4 expression. In the manual PCR, RBBP4 expression appeared to be the same in MDA-MB-231 and MDA-MB-468, whereas in the qPCR there was double the amount of RBBP4 in MDA-MB-468.

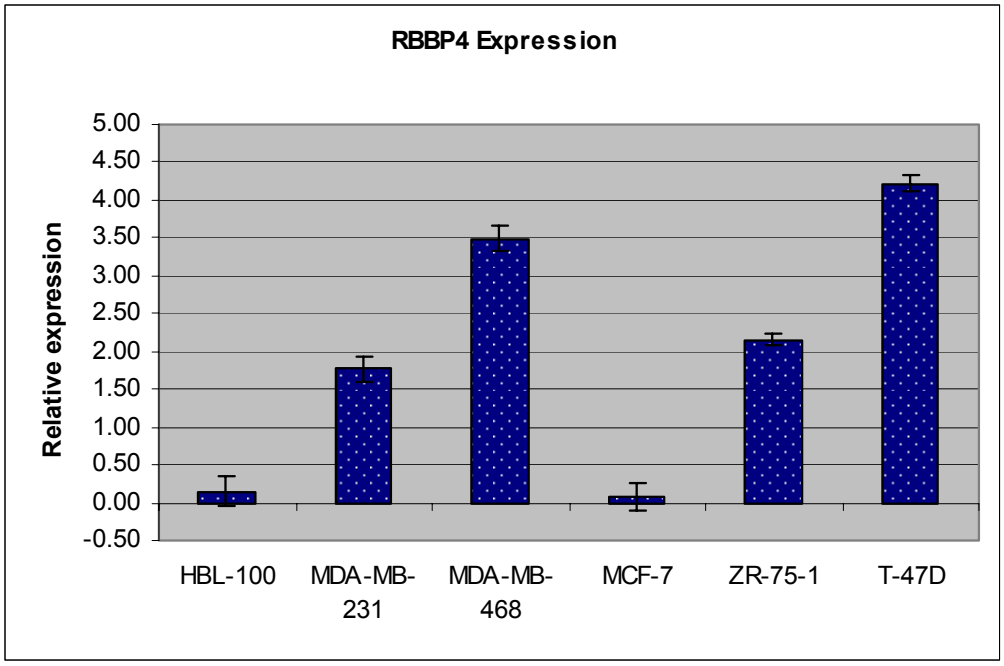
Figure 3.17 RBBP4 Expression in cell lines

A.



B.

A.



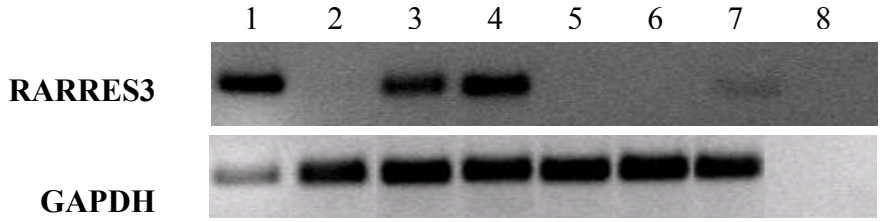
RBBP4 expression in 6 cell lines. The bottom gel picture shows GAPDH expression in the same cell lines. Lanes: 1, 100bp DNA ladder ; 2, HBL-100; 3, MDA-MB-231; 4, MDA-MB-468; 5, MCF-7; 6, ZR-75-1; 7, T-47-D; 8 , Water Blank. B: Relative RBBP4 expression in 6 cell lines using RT-qPCR. RT-qPCR experiments were carried out in triplicate (n=18). Error bars reflect standard error of the mean (SEM).

3.3.8 RARRES3 Expression

Manual PCR found weak RARRES3 expression in T47-D, moderate expression in MDA-MB-231, and a high expression in MDA-MB-468 (see Figure 3.18 A). Although the RT-qPCR found high expression of RARRES3 in MDA-MB-468, and low expression in T47-D, expression in MDA-MB-231 was much lower than observed by manual PCR. No RARRES3 expression was found in MCF-7 and ZR-75-1.

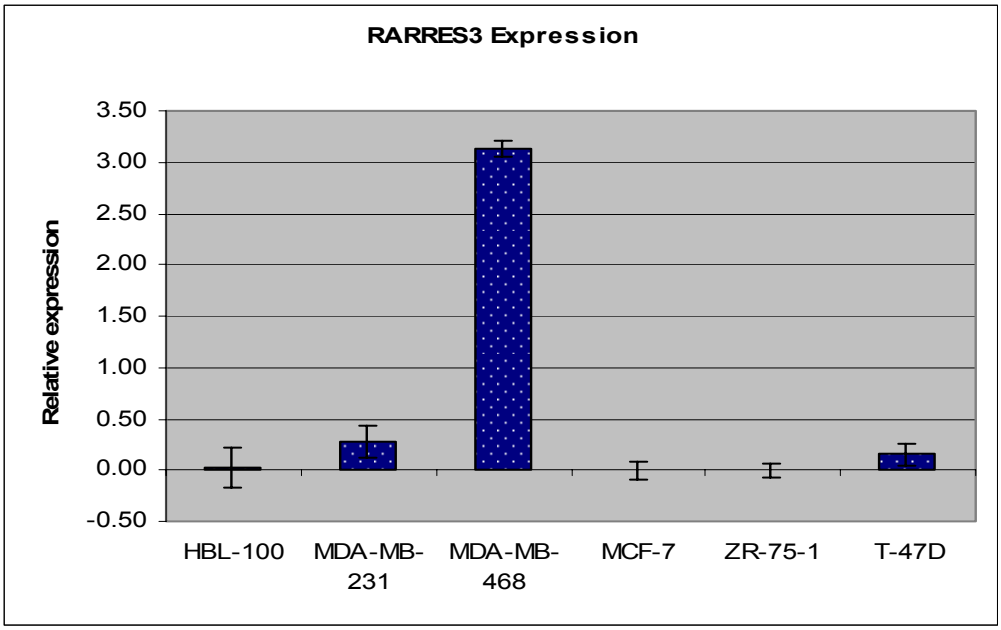
Figure 3.18 RARRES3 Expression in cell lines

A.



B.

A.



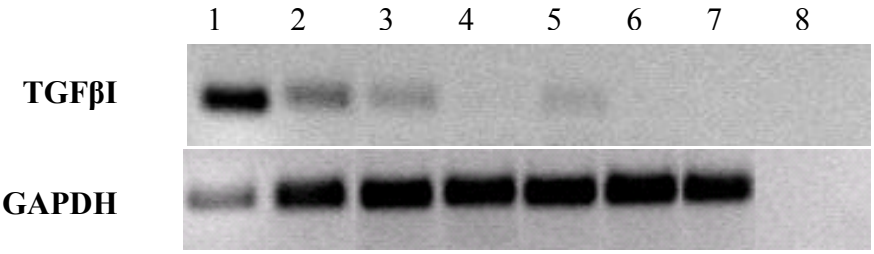
RARRES3 expression in 6 cell lines. The bottom gel picture shows GAPDH expression in the same cell lines. Lanes: 1, 100bp DNA ladder ; 2, HBL-100; 3, MDA-MB-231; 4, MDA-MB-468; 5, MCF-7; 6, ZR-75-1; 7, T-47-D; 8 , Water Blank. B: Relative RARRES3 expression in 6 cell lines using RT-qPCR. RT-qPCR experiments were carried out in triplicate (n=18). Error bars reflect standard error of the mean.

3.3.9 TGFβI Expression

Both manual PCR and real-time qPCR found strong TGFβI expression in HBL-100, and lower levels in MDA-MB-231, and MCF-7 cells (see Figure 3.19 A and B) only. Again these results were different to the array, where TGFβI was up-regulated in the 2 tumours.

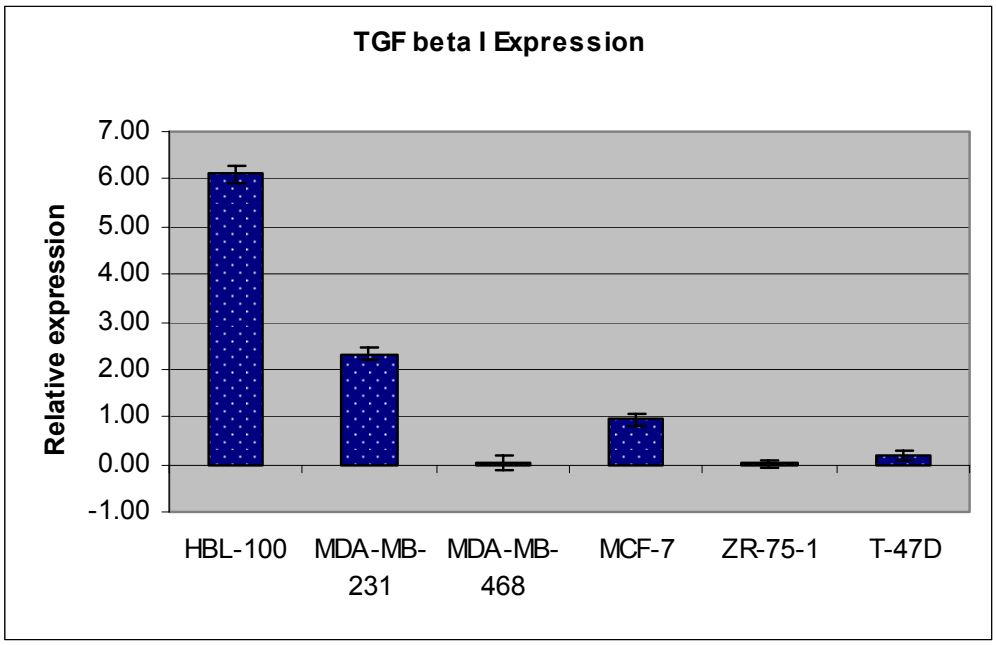
Figure 3.19 TGFβI Expression in cell lines

A.



B.

A:



TGFβI expression in 6 cell lines. The bottom gel picture shows GAPDH expression in the same cell lines. Lanes: 1, 100bp DNA ladder ; 2, HBL-100; 3, MDA-MB-231; 4, MDA-MB-468; 5, MCF-7; 6, ZR-75-1; 7, T-47-D; 8 , Water Blank. B: Relative TGFβI expression in 6 cell lines using RT-qPCR. RT-qPCR experiments were carried out in triplicate (n=18). Error bars reflect standard error of the mean.

3.3.10 Summary

The microarray found all 9 target genes to be overexpressed in the 2 breast tumours from women ≤ 35 years in comparison to normal organoids and the HBL-100 breast cell line. Although immortalised cell lines represent only a 2-D model of breast cancer and are each very different in behaviour, the analysis of target gene expression in 6 breast cell lines using RT-qPCR in this study appeared to support the microarray with high levels of expression of 7 out of 9 target genes in the breast cancer cells and lower levels in the non-tumourigenic HBL-100 (for a summary see Table 3.7). In contrast however, C/EBPα and TGFβI were both found to have higher levels of expression in the non-tumourigenic HBL-100 compared to the breast cancer cell lines.

Table 3.7 Summary of target gene expression in 6 breast cell lines

Target Gene	Array	Cell Lines						Difference between ER-pos/neg cells
		HBL-100	231	468	MCF-7	ZR-75-1	T47-D	
AKAP1	+	–	+	–	+	+	+	NO
APRIL	+	–	+	+	–	–	–	YES
C/EBPα	+	+	–	–	–	–	–	NO
DDB2	+	–	–	–	+	+	+	YES
GRANULIN	+	–	+	+	+	+	+	NO
NCOA3	+	–	–	–	+	+	+	YES
RARRES3	+	–	–	+	–	–	–	NO
RBBP4	+	–	+	+	–	+	+	NO
TGFβI	+	+	+	–	–	–	–	NO

Relative expression shown with an arbitary cut off of 1.5. Cells with relative expression values < 1.5: −, those with relative expression values > 1.5: +

3.4 Target gene expression in reduction mammoplasty tissue

Normal breast organoids, isolated following digestion of breast reduction tissue consist of myoepithelial and luminal cell populations that are present in the mammary gland. Organoids were isolated by digestion of tissue from a total of 9 reduction mammoplasty specimens (see section 2.2.2 and Table 3.8). These served as an important relative control for the subsequent target gene expression analysis in tumours since they reflect the behaviour of normal breast epithelial cells *in vivo*. In addition, most specimens were from young women (age range was 20-41, with mean age of 34.2 years) ideal for comparison with tumours from young women (≤35 yrs). Menstrual cycle data was not available.
RNA was isolated from the organoids (see section 2.2.3), and cDNA was prepared as before. Real-time qPCR was then performed on all 9 samples using primer sets for each of the nine target genes. All data is represented as relative expression levels: target gene expression relative to the average of the 3 housekeeping genes (Target/Housekeeping gene).

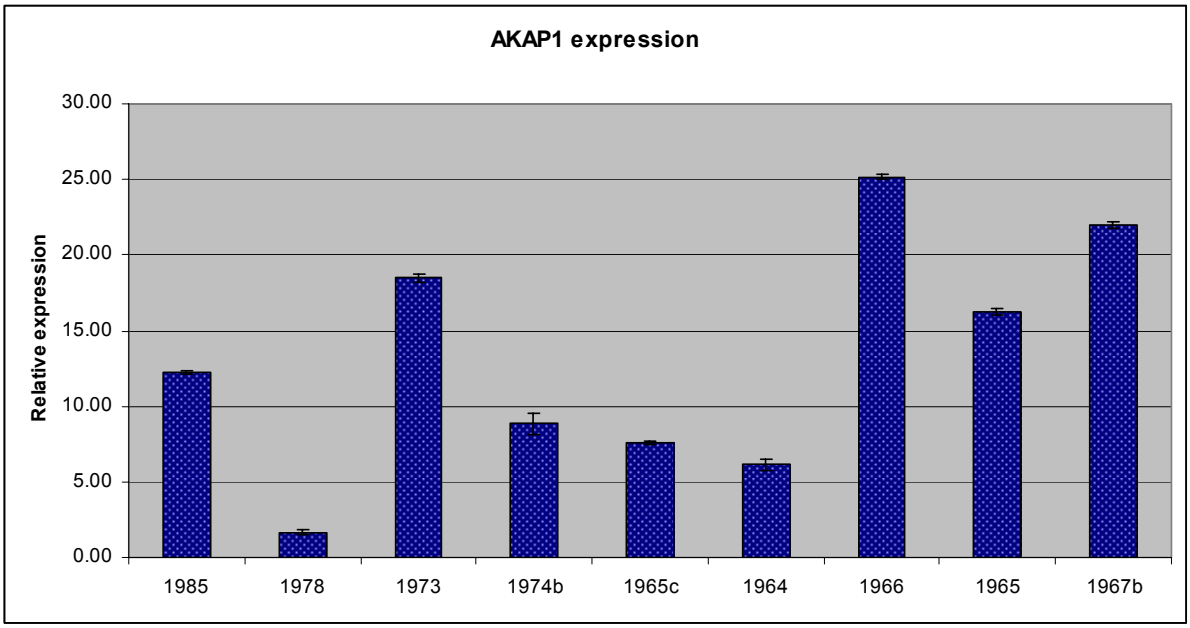
Table 3.8 Summary of reduction mammoplasty specimen details

Sample number	Age
1985	20
1978	27
1973	32
1974b	31
1965c	40
1964	41
1966	39
1965	40
1967b	38
Mean	34.2
Range	20-41

3.4.1 AKAP1 expression

Real-time qPCR found variation in AKAP1 expression between the different donors (see Figure 3.20). However all 9 samples had AKAP1 levels greater than that of the average of the 3 housekeeping genes. Relative expression ranged from 2.38 to 25.15 (mean= 13.15, median =12.24).

Figure 3.20 AKAP1 expression in 9 normal breast organoids

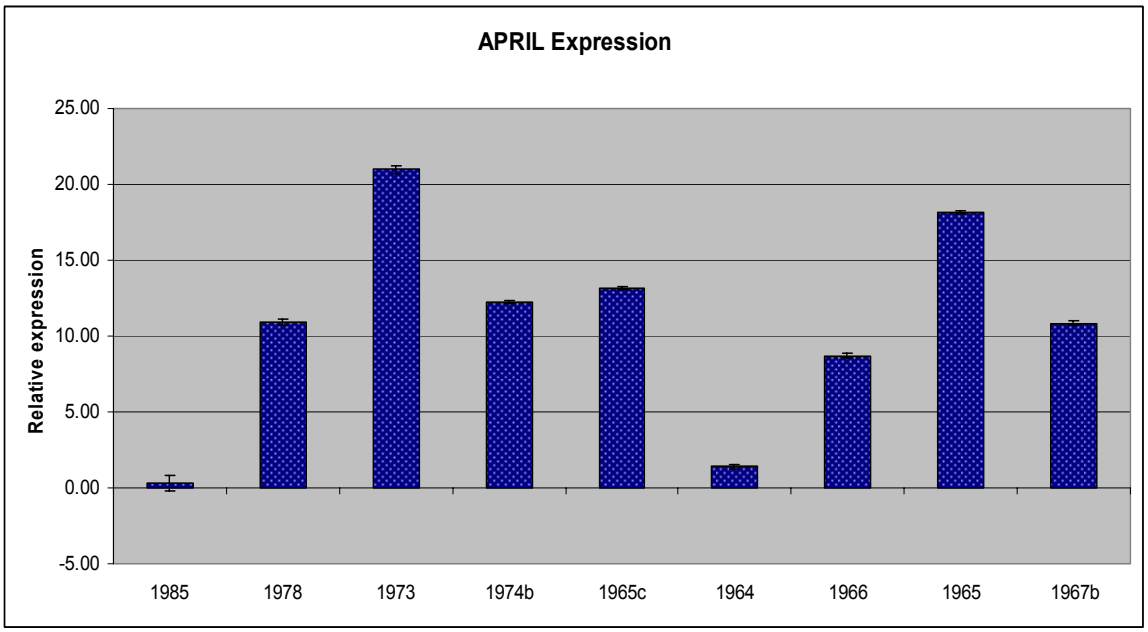


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=27).

3.4.2 APRIL expression

APRIL showed variation in expression between the organoid samples (see Figure 3.21), with most samples displaying high levels of expression. Seven out of the nine samples tested had relative expression levels above 7, with the highest approaching 22. Two samples had relative expression levels less than 1.42. The mean and median expression levels were 10.74, and 10.88 respectively.

Figure 3.21 APRIL Expression in 9 normal breast organoids

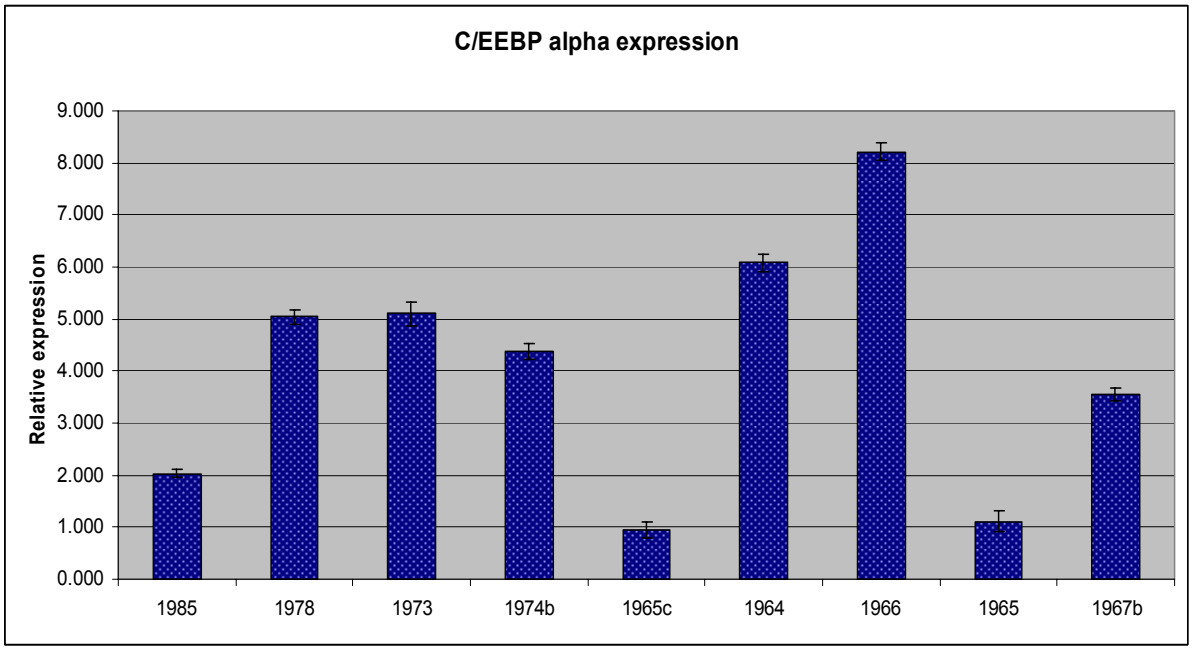


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=27).

3.4.3 C/EBP alpha expression

Almost all organoids samples showed strong C/EBP alpha expression, with fold changes averaging 4.05 (Figure 3.22). Two samples showed C/EBP alpha expression similar to the housekeeping genes (#1965, and 1965c). The mean and median expression levels were 4.05, and 4.38 respectively.

Figure 3.22 C/EBP alpha expression in 9 normal breast organoids

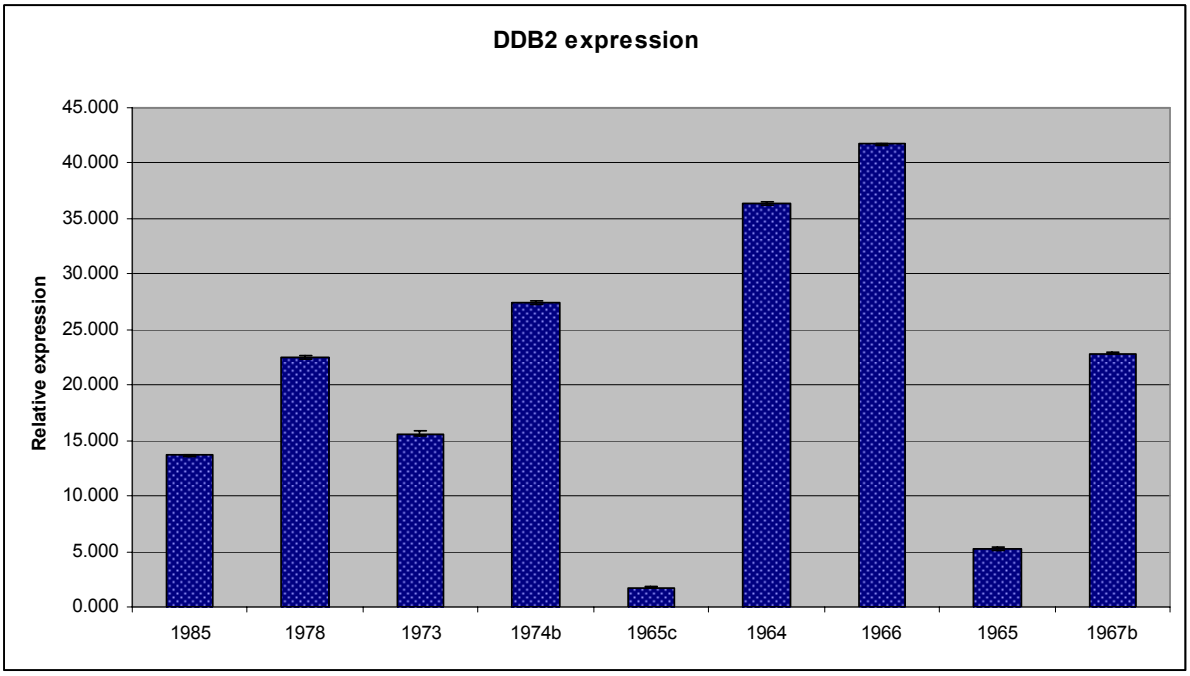


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=27).

3.4.4 DDB2 expression

DDB2 expression was high for 8 of 9 organoids samples (see Figure 3.23). Relative expression levels ranged from 1.71 to 41.6 (mean = 20.78, and median = 22.51).

Figure 3.23 DDB2 Expression in 9 normal breast organoids

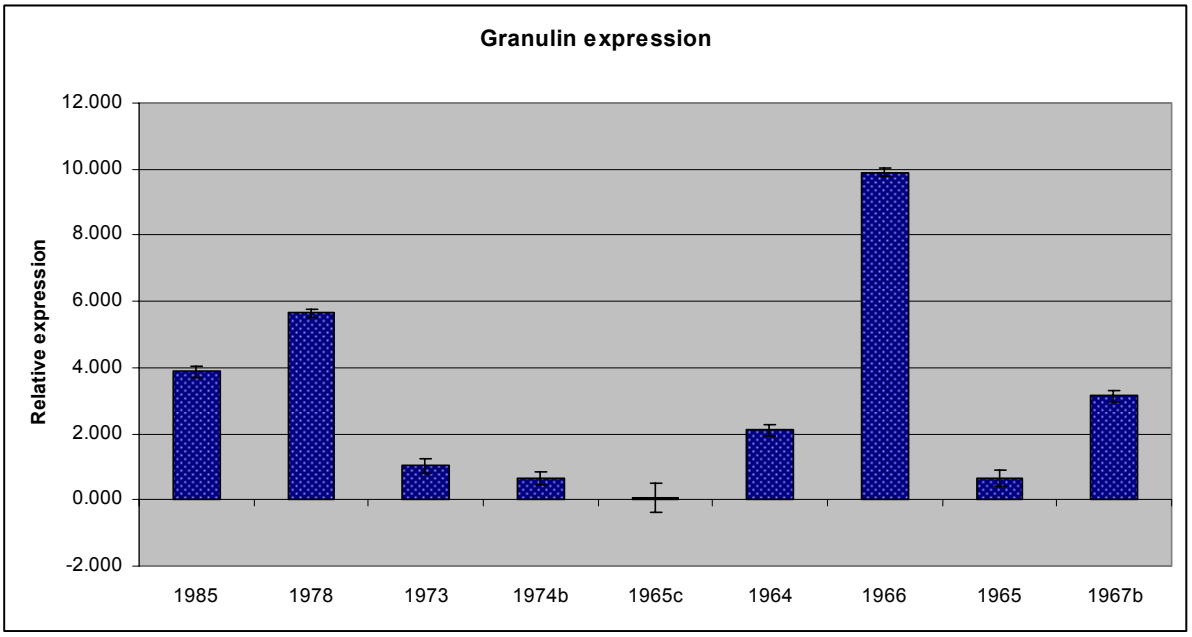


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=27).

3.4.5 Granulin expression

Granulin expression varied widely in the organoid samples (see Figure 3.24); samples 1965, 1974b, 1965c and 1973 had relative expression levels less than 2, while the 5 others ranged from 2 to 10 (mean = 3.01, and median = 2.11).

Figure 3.24 Granulin expression in 9 normal breast organoids

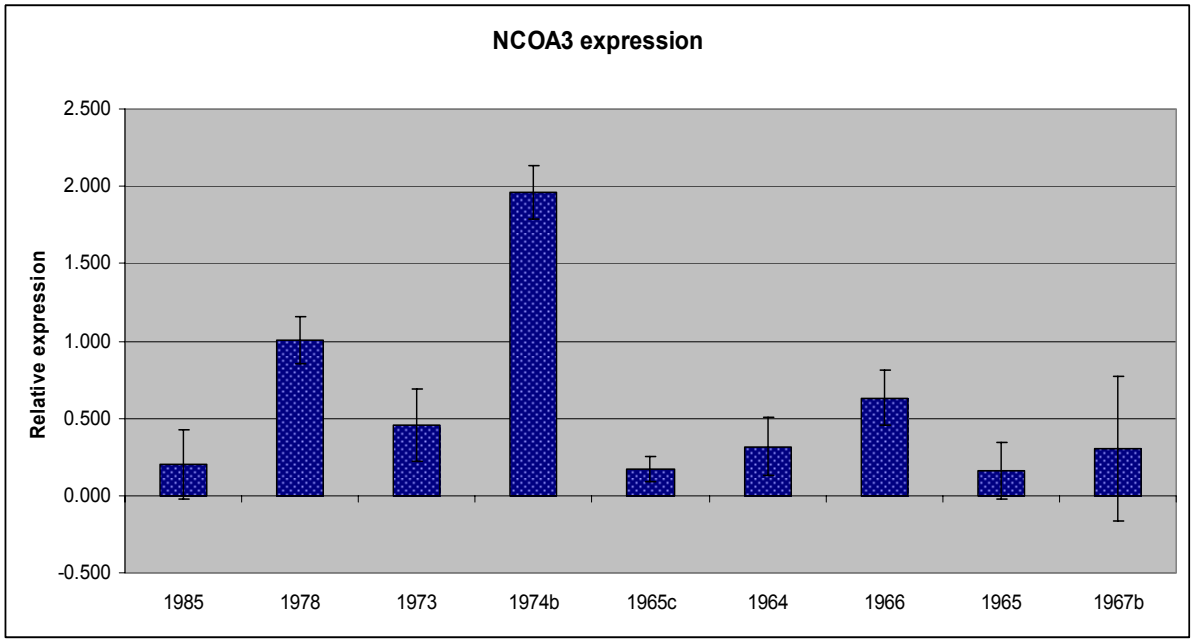


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=27).

3.4.6 NCOA3 expression

NCOA3 expression varied greatly in the organoids (see Figure 3.25). In general the expression was low compared to the expression of housekeeping genes with 7/9 samples showing relative expression ratios below 1. Mean and median expression levels were 0.58, and 0.32 respectively.

Figure 3.25 NCOA3 expression in 9 normal breast organoids

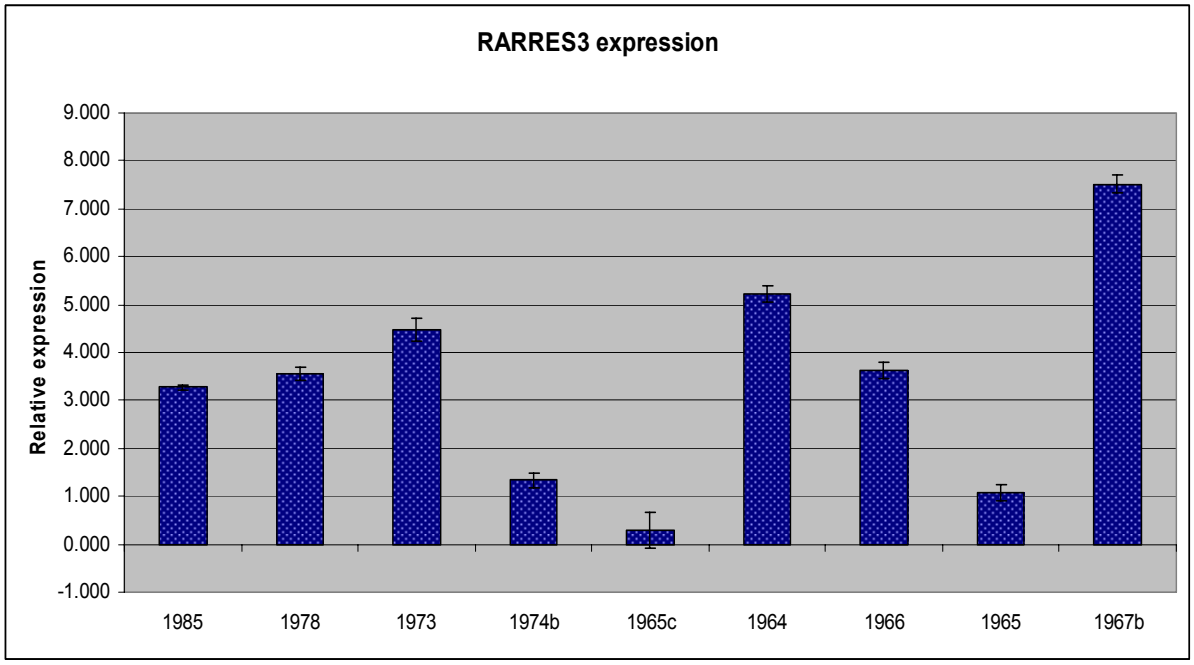


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=27).

3.4.7 RARRES3 expression

RARRES3 expression was more than double that of the housekeeping genes in six out of the nine organoids samples (see Figure 3.26). The remaining 3 samples had relative expression values below 1.34. The mean RARRES3 expression level was 3.37, and median 3.54.

Figure 3.26 RARRES3 expression in 9 normal breast organoids

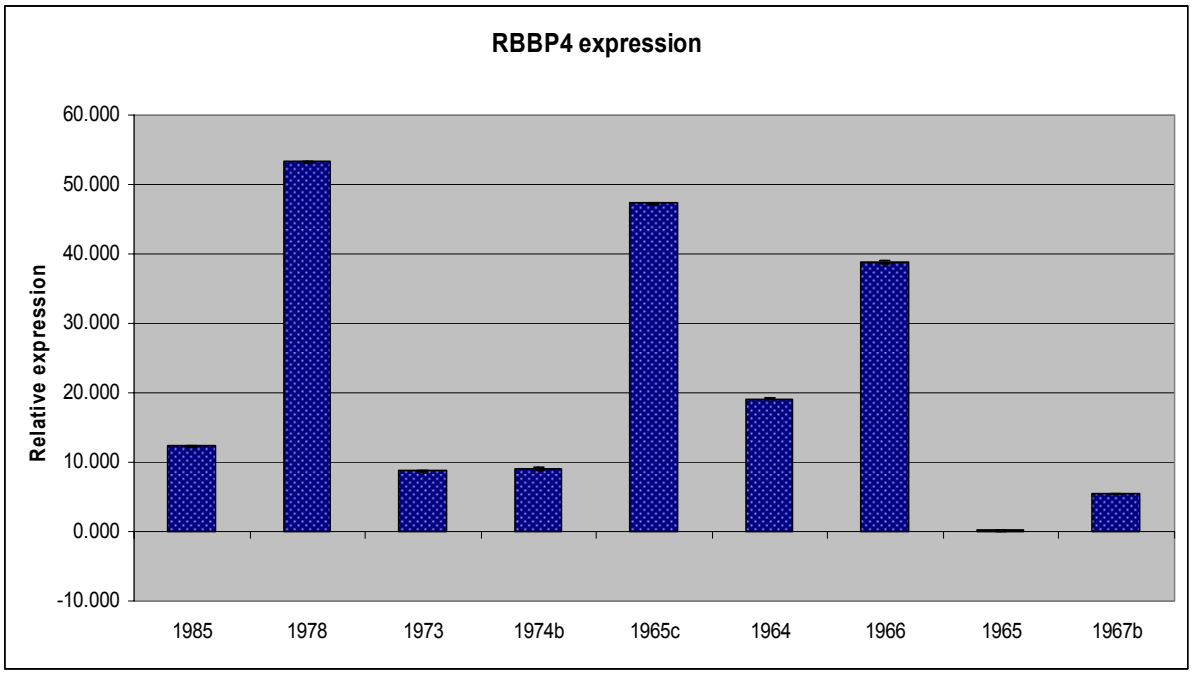


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=27).

3.4.8 RBBP4 expression

RBBP4 showed very high expression across all organoids samples with the exception of one (#1965 fold change of 0.15) (see Figure 3.27). In the positive samples, relative expression figures ranged from 5.5 for a 38 year old to 53.2 for a 27 year old (# 1967b and 1978). The average relative expression was 21.58, and median was 12.31.

Figure 3.27 RBBP4 expression in 9 normal breast organoids

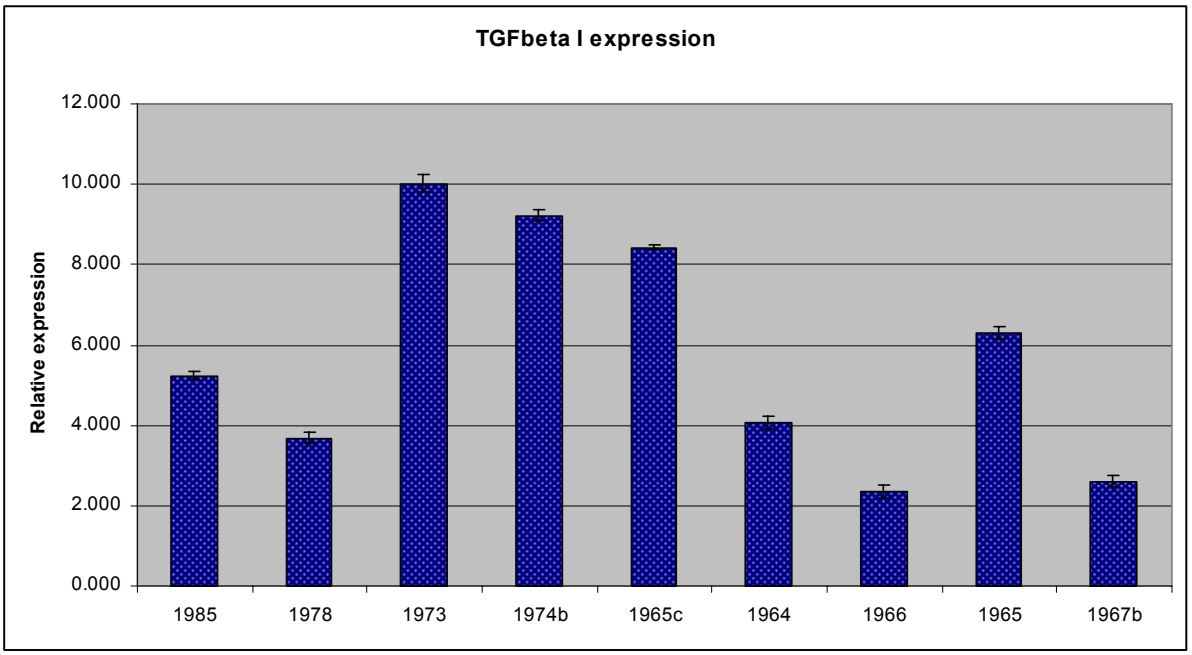


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=27).

3.4.9 TGFβI expression

TGFβI expression was high among all organoids samples (see Figure 3.28). The relative expression of TGFβI ranged from 2.35 to 10 (mean = 5.76, and median = 5.23).

Figure 3.28 TGF beta I expression in 9 normal breast organoids



Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=27).

3.4.10 Summary of expression data for 9 normal organoid samples

Overall, there was considerable variation in the expression of the 9 target genes in the 9 organoids samples. The expression of 8/9 targets was higher in the organoids samples than the mean of the 3 housekeeping genes (see Table 3.9). Only NCOA3 showed low levels of expression in the organoids. Incidentally, individual organoids samples showed variations in expression between genes eg. 1985 and 1965c. This shows that there were no significant problems with RNA quality. The expression levels of 7/9 target genes were much higher in the organoids samples than in the non-tumouriogenic HBL-100, with the exception of NCOA3 and TGFβI where levels appeared higher in the HBL-100 cell line. In the microarray, the expression of all nine target genes was high in tumours when either the organoids or HBL-100 samples were used as the normaliser.

Table 3.9 Summary of mean target gene expression in HBL-100 and organoids samples

Gene	Relative expression	
	Organoids	**HBL-100**
AKAP1	13.15	0.81
APRIL	10.74	0.16
C/EBPα	4.05	1.94
DDB2	20.78	1.03
Granulin	3.01	0.54
NCOA3	0.58	0.74
RARRES3	3.37	0.02
RBBP4	21.58	0.15
TGFβI	5.76	6.11

Organoids: mean relative expression of all 9 samples. The expression of 8/9 targets was higher in the organoids samples than the mean of the 3 housekeeping genes.

3.5 Target gene expression in tumour samples

A total of 21 snap frozen tumours (11 cases >35 years, and 10 case <35 years) were available for qPCR analysis (see Table 3.10). One 4 μM section of each tumour tissue was first stained with hematoxylin and eosin (H&E) to verify that adequate numbers of invasive tumor cells were present. All tumour cases were estimated to contain >80% tumour cells (verified by Prof. R.A. Walker). Subsequently, ten 4μM thick sections were cut from each block and RNA was extracted (see section 2.3.3.1). Three micrograms of total RNA was converted to cDNA using reverse transcriptase as outlined in sections 2.2.3.1 and 2.2.7.1. The cDNA was then examined for the expression of the nine target genes using SYBR Green real-time quantitative PCR (see section 2.2.8). Each experiment was run in triplicate and all results were represented as relative expression (target gene expression relative to the average of the 3 housekeeping genes (Target/Housekeeping gene). Statistical analysis was performed to determine whether or not there were any significant differences in the level of expression of each target gene in each the age group.

Table 3.10 Patient details for snap frozen tissues

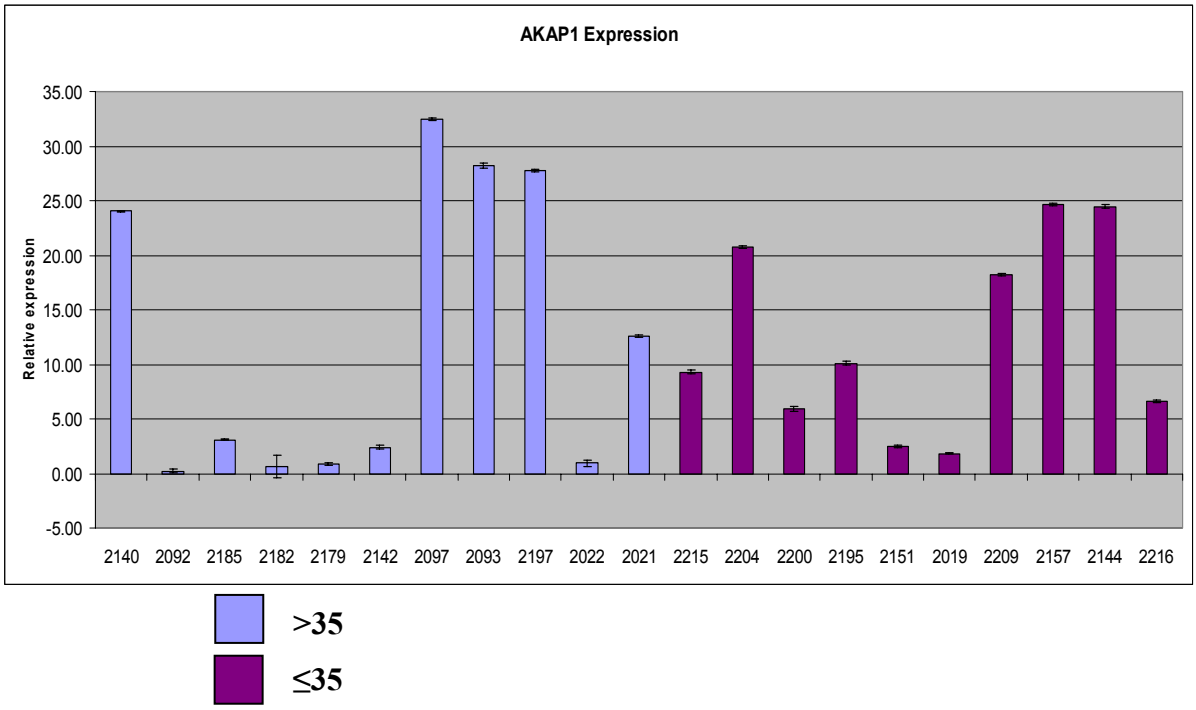
RW No	Age	Size	Type	Grade	Node	ER	PgR
2157	19	1	IDC	III	Neg	Pos	Pos
2195	22	2	IDC	III	Pos	Neg	Neg
2215	25	2	IDC	III	Pos	Pos	Pos
2151	28	2	IDC	III	Neg	Neg	Neg
2144	30	2	IDC	III	Neg	Neg	Neg
2019	31	1	IDC	III	Pos	Pos	Pos
2204	29	1	IDC	III	Neg	Pos	Pos
2200	34	2	IDC	III	Pos	Neg	Pos
2209	35	1	IDC	III	Neg	Neg	Neg
2216	35	2	IDC	II	Pos	Pos	Pos
2140	38	2	IDC	II	Neg	Pos	Pos
2142	50	1	IDC	III	Pos	Pos	Pos
2197	50	2	IDC	II	Neg	Pos	Pos
2185	51	2	IDC	III	Pos	Neg	Neg
2179	51	2	IDC	II	Pos	Pos	Pos
2097	54	2	IDC	III	Pos	Pos	Pos
2021	54	2	IDC	III	Pos	Pos	Pos
2092	59	1	IDC	III	Neg	Neg	Neg
2093	62	2	IDC	III	Neg	Pos	Pos
2182	64	2	IDC	III	Neg	Neg	Neg
2022	64	2	ILC	III	Pos	Pos	Pos

Size - 1: tumour 20 mm or less; 2: tumour 20-50mm in size
IDC: Infiltrating ductal carcinoma; ILC: infiltrating lobular carcinoma
Neg: Negative Pos: Positive;
ER and PgR information was obtained from NHS patient records.
Blank, are where patient information was not available

3.5.1 AKAP1 expression

AKAP1 was up regulated in the two tumour cases examined by microarray (fold change =3.98). Using qPCR analysis of snap frozen tumour tissue AKAP1 was shown to have a higher expression compared to that of the 3 housekeeping genes, with the exception of 4 samples in the >35 years old group (see Figure 3.29). Relative expression of AKAP1 ranged from 0.21 to 32.4. There were more grade III than grade II cases but there were no differences in expression between them. Although tumours from women ≤35 years appeared to have a higher relative expression compared to those >35 years (median relative expression levels for the ≤35 years and >35 years were 9.68 and 3.08 respectively), AKAP1 expression did not differ significantly between the two age groups (p=0.557). AKAP1 expression also did not appear to follow a pattern with respect to grade or node status.

Figure 3.29 AKAP1 expression in 21 snap frozen tumour cases

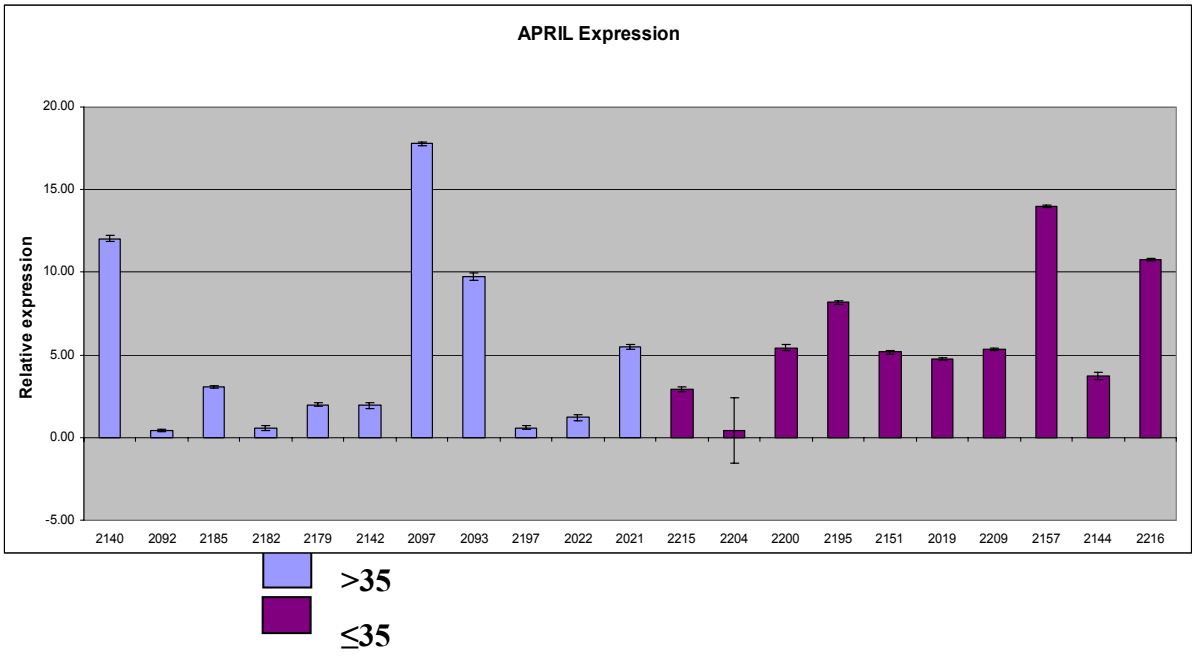


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=63).

3.5.2 APRIL expression

APRIL expression was high in the snap frozen tumour samples; with the exception of 6 tumour cases >35 years (see Figure 3.30). Tumours from women ≤35 years generally had higher levels of AKAP1 expression which is in support of the findings of the microarray (fold change 2.23. The relative expression levels ranged from 0.4 to 17.7, and the mean and median were 4.98 and 1.98 for tumours from women >35 years and 6.07 and 5.26 for those from women ≤35 years. In the >35 years group, 7 samples were found to have relative expression levels above the median value, while 4 samples showed expression levels that were above the mean value. Despite tumours from women ≤35 years appearing to have generally higher levels of AKAP1 in comparison to the older age group, when the data is analysed using ANOVA, there was no significant difference between the expression profiles of the two age groups (p=0.823). Analysis of APRIL expression in breast tumour cell lines saw a higher level of expression in ER-negative cell lines. The same trend was not observed in tumour samples. Although only 2 (both >35 years) out of a total of 8 samples that were ER-negative had relative expression levels less than 1.00, in the remaining 13 ER-positive samples, 11 had relative expression levels above 1.00. No relationship was seen with grade or node status.

Figure 3.30 APRIL expression in 21 snap frozen tumour cases

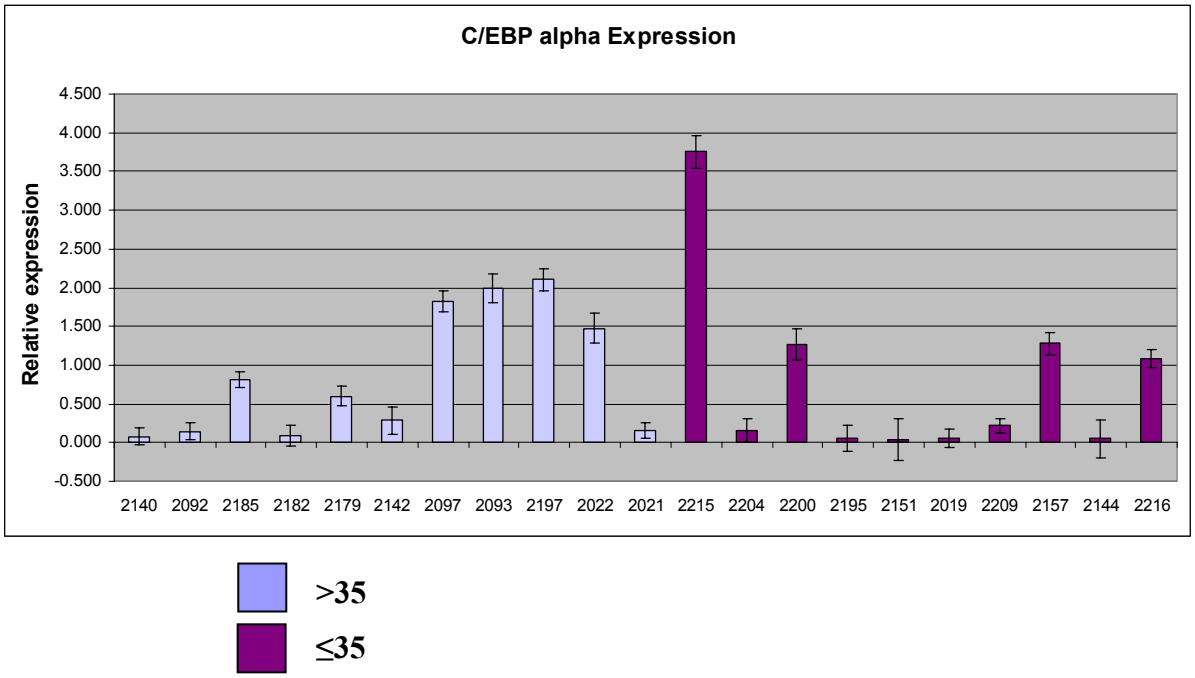


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=63).

3.5.3 C/EBP alpha expression

The results of C/EBP qPCR analysis of snap frozen tumours found almost all tumour cases to have fold changes below 2 (see Figure 3.31). These findings are in contrast to the microarray analysis (fold change was 2.96). The overall range of relative expression was 0.04 to 3.75. In tumours from women >35 years, 7 cases had relative expression levels below the mean value (0.87) while 5 cases had relative expression levels below the median values (0.60). In tumours from women ≤35 years, the mean and median values were 0.80 and 0.19 respectively. As for the samples >35 years, the majority of tumour cases from women ≤35 years had relative expression levels below the mean and median levels. No difference between the expression profiles of the age groups was seen (p=0.941). No relationship was seen with grade or node status.

Figure 3.31 C/EBP alpha expression in 21 snap frozen tumour cases

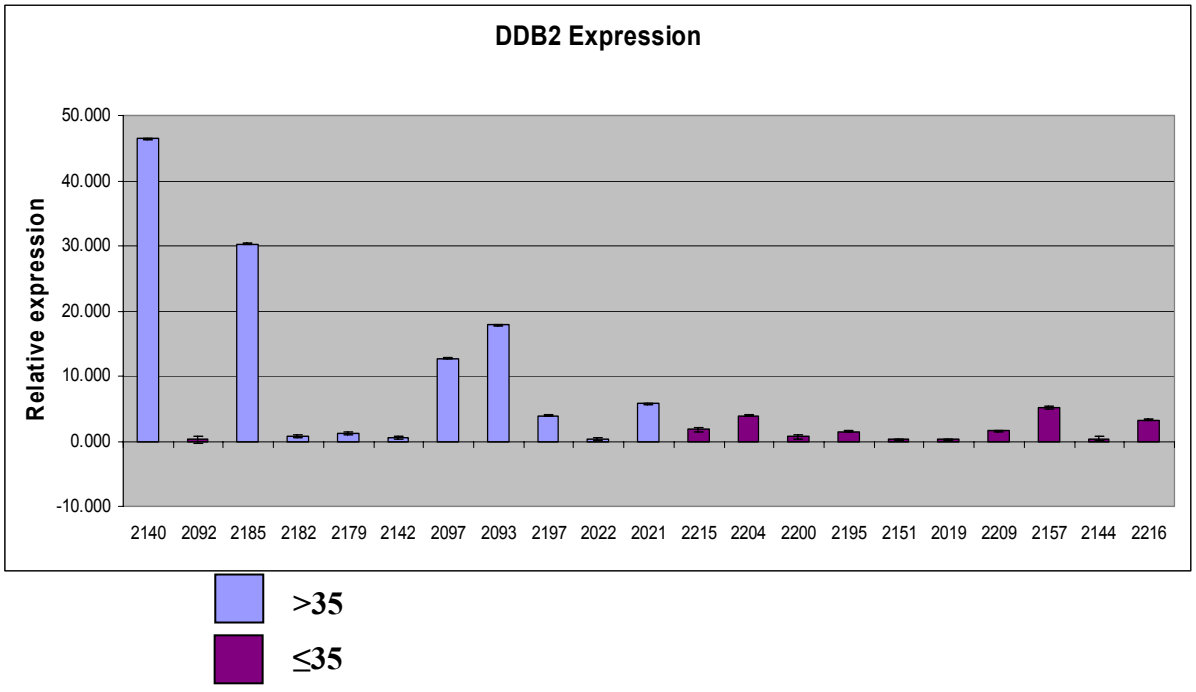


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=63).

3.5.4 DDB2 Expression

There was great variation in DDB2 expression among snap frozen tumour samples from tumours from women >35 years, while little variation was seen in tumour samples from women <35 years (see Figure 3.32). The relative expression levels for samples in the >35 years old group ranged from 0.26 to 46.30, with mean and median levels at 10.89 and 3.93 respectively. Four of these samples had relative expression levels greater than the mean while 6 samples showed relative expression levels greater than the median. Tumour samples in the <35 years old group had relative expression levels ranging from 0.22 to 5.10 and the mean and median levels were 1.87 and 1.55 respectively. The majority of samples in this group had relative expression levels below that of the mean or median. There was no statistical difference between the amount of DDB2 expression in both age groups (p=0.355). Despite a higher DDB2 expression in ER-positive breast tumour cell lines, no clear difference in its expression in tumour samples with respect to ER status was seen. Although 10 out of 13 ER-positive tumour cases had relative expression levels above 1.00, in the remaining 8 ER-negative samples, 3 had relative expression levels above 1.00. There was no apparent relationship between DDB2 expression and either grade or node status.

Figure 3.32 DDB2 expression in 21 snap frozen tumour cases

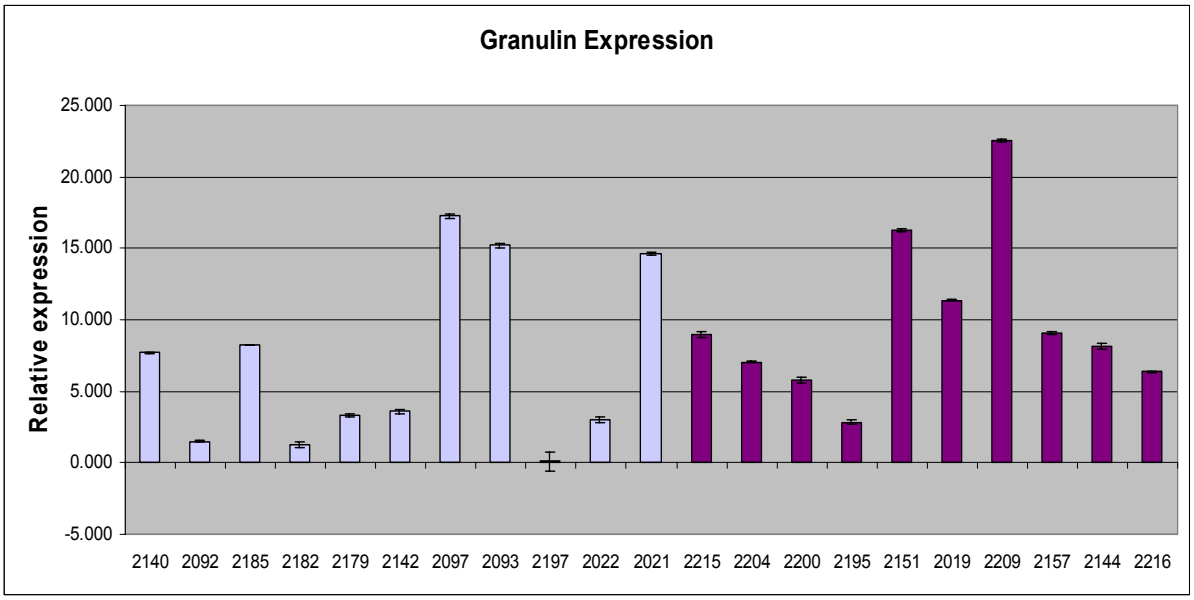


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=63).

3.5.5 Granulin expression

Real time qPCR analysis of granulin showed expression levels across the majority of snap frozen tumour specimens to be higher than those of the 3 housekeeping genes (see Figure 3.33). Relative expression levels for samples in the ≤35 years group ranged from 2.8 to 22, while the mean and median were 8.56 and 3.59 respectively. In the >35 years old group relative expression levels ranged from 0.1 to 17, and the mean and median were 9.86 and 6.89 respectively. The results here support the microarray (fold change of 4.11). No differences were seen between different grades, or node status. Again, there was no difference in the expression of granulin between both age groups (p=0.42).

Figure 3.33 Granulin expression in 21 snap frozen tumour cases

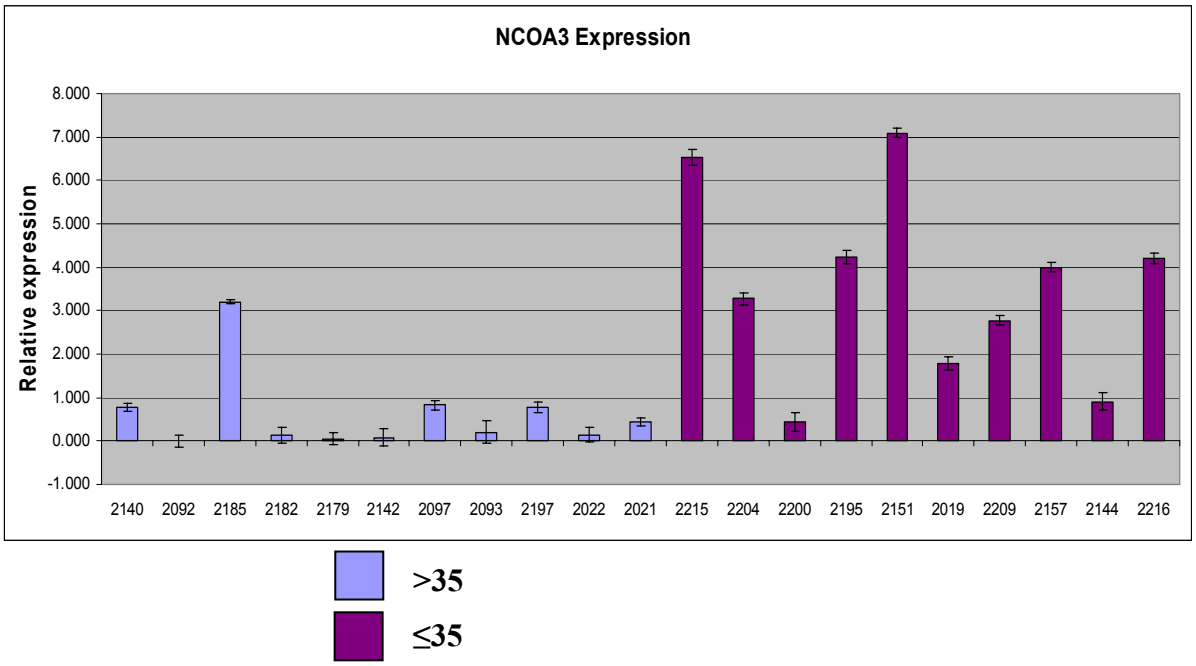


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=63).

3.5.6 NCOA3 expression

SYBR Green qPCR analysis of NCOA3 expression of snap frozen tumour specimens found a higher expression among tumours from women ≤35 years old (relative expression levels ranged from 0.44 to 7.00), compared to those >35 years old (relative expression levels ranged from 0.001 to 3.20) (p=0.001) (see Figure 3.34). Only one case from the older age group (#2185) showed NCOA3 expression greater than those of the 3 housekeeping genes. The mean and median relative expression levels for tumours from women >35 years were 0.60 and 0.20 respectively, whereas those for tumours from women ≤35 years were 3.53 and 3.64 respectively. These results support the findings of the microarray (fold change of 5.33). Analysis of NCOA3 expression in breast tumour cell lines saw a higher level of expression in ER-positive cell lines. The same trend was not observed in tumour samples. In tumours from women ≤35 years, 5 samples with a relative expression greater than 1 were ER-positive, 3 with relative expression above 1 were ER-negative, and 2 samples with relative expression below 1 were ER-negative. In tumours from women >35 years, 1 sample with a relative expression above 1 was ER-negative, 8 with a relative expression below 1 were ER-positive, while 2 samples with a relative expression below 1 were ER-negative. The difference in NCOA3 expression between the two age groups was statistically significant.

Figure 3.34 NCOA3 expression in 21 snap frozen tumour cases

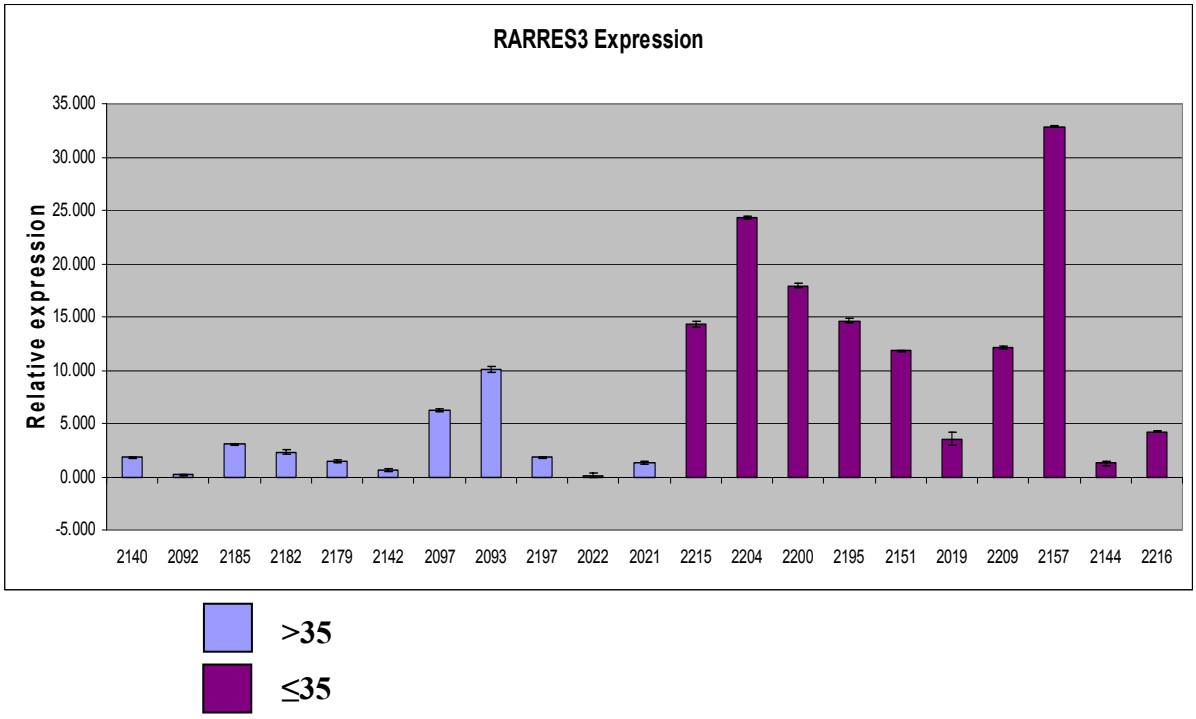


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=63).

3.5.7 RARRES3 expression

Real time qPCR analysis of RARRES3 expression of snap frozen tumours specimens revealed expression levels to be greater then those of the 3 housekeeping genes in tumours from women ≤35 years (relative expression ranged from 1.2 to 32.6; mean = 13.70, median=13.25) (see Figure 3.35). Expression levels in tumours from women >35 years were lower (relative expression ranged from 0.12 to 10; mean=2.62, median=2.78). The results here support the findings of the microarray (fold change of 3.42). No relationship was found between RARRES3 expression and grade or ER/PgR status. However there was a difference in RARRES3 expression between the two age groups (p=0.002).

Figure 3.35 RARRES3 expression in 21 snap frozen tumour cases

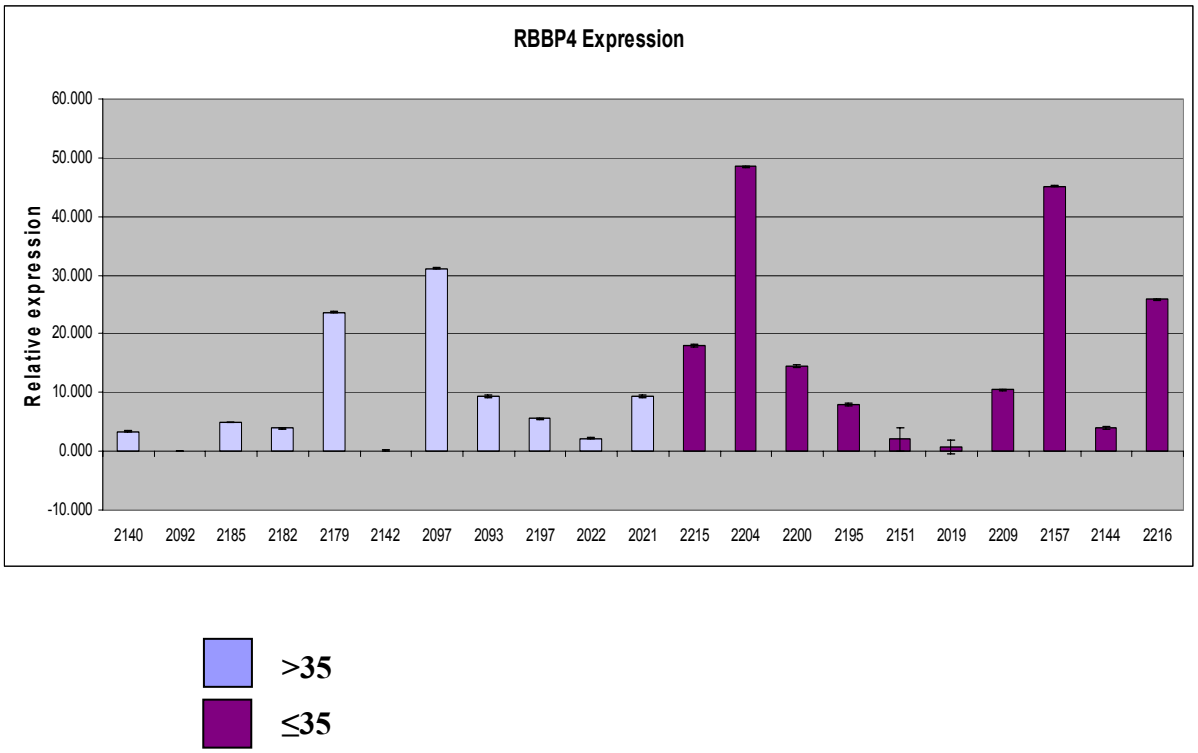


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=63).

3.5.8 RBBP4 expression

In snap frozen tumour tissues the expression of RBBP4 was higher than those of the 3 housekeeping genes across most samples, with fold changes ranging from 0.03 to 23.60 for cases >35 years (mean= 8.51, and median= 4.94) and 0.70 to 48.53 for those ≤35 years (mean= 17.74, and median=12.51) (see Figure 3.36). No difference in the expression between the two age categories was observed (p=0.446). Similarly, no difference was seen between RBBP4 expression and grade or ER/PgR status.

Figure 3.36 RBBP4 expression in 21 snap frozen tumour cases

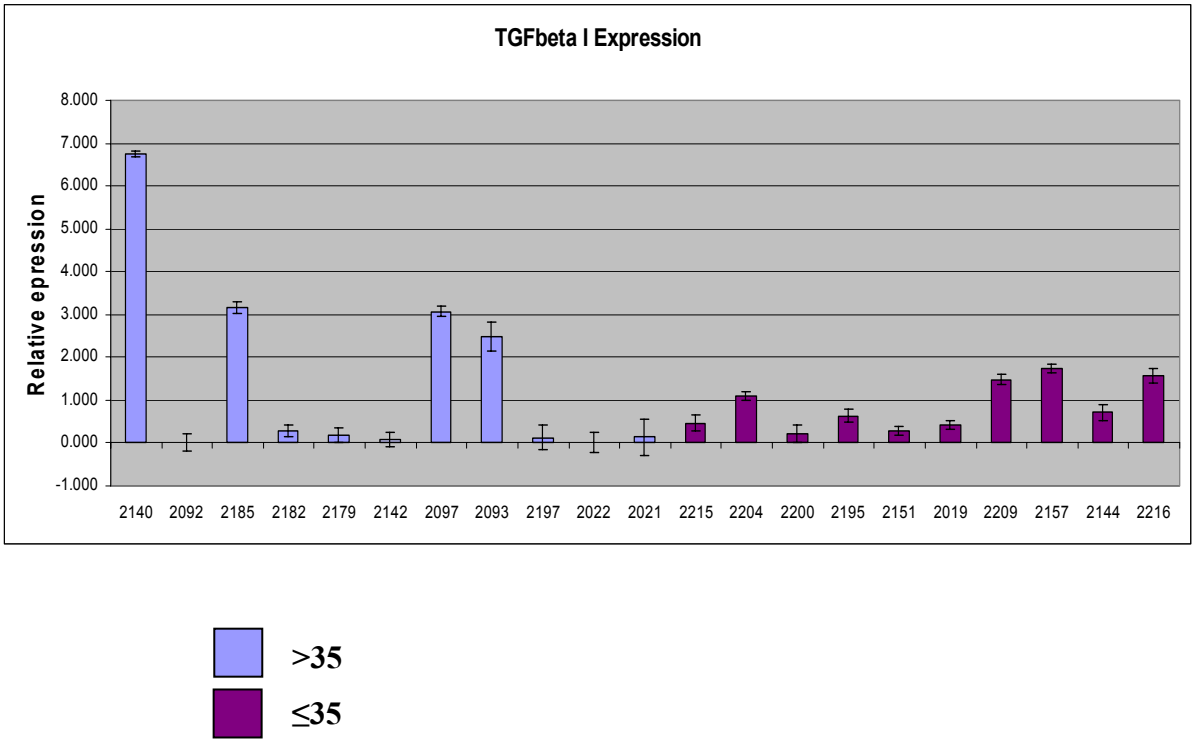


Error bars reflect standard error of the mean (SEM). RT-qPCR experiments were carried out in triplicate (n=63).

3.5.9 TGFβI expression

TGFβI relative expression was below 2 in the majority of snap frozen tumour tissues (see Figure 3.37), with the exception of a 4 cases >35 years. In the>35 years group, relative expression ranged from 0.003 to 6.70 (mean= 1.48, median= 0.86), and in the ≤35 years group relative expression ranged from 0.22 to 1.73 (mean= 0.86, median=0.67). These results are in contrast to the microarray study which found a fold change of 3.53 in breast tumours ≤35 years. There was no significant difference between the expression levels in both age groups (p=0.566). TGFβI expression in the tumour samples in general did not differ between tumours of differing grades or node status.

Figure 3.37 TGFβI expression in snap frozen tumour cases



Error bars reflect standard error of the mean. RT-qPCR experiments were carried out in triplicate (n=63).

3.5.10 Summary of gene expression changes in 21 breast tumours

Target gene expression analysis in snap frozen tumour tissues showed considerable variability in the expression levels of each target gene in all tumour samples (≤35 and >35) (see Table 3.11). Seven out of nine target genes showed high expression levels in breast cancers from younger women lending support to the microarray data. Of the seven, two of these target genes (NCOA3 and RARRES3) had significant different levels of expression between the two age groups. In contrast to the microarray, C/EBPα, and TGFβI showed predominantly low levels of expression in all tumour samples.

In breast cancer cell lines, NCOA3, APRIL, and DDB2 were found to have expression levels differing between ER-positive an ER-negative cells. In the case of the tumour specimens, this pattern was not observed. High levels of NCOA3, APRIL, and DDB2 were seen in both ER-positive and ER-negative samples. Similarly target gene expression did not follow a trend based on the node status of the patient.

Table 3.11 Summary of target gene expression in snap frozen tumour tissues

Target gene	>35 years	≤35 years
AKAP1	↑	↑
APRIL	↑	↑
C/EBPα	↓	↓
DDB2	↑	↑
GRANULIN	↑	↑
NCOA3	↓	↑
RARRES3	↑	↑
RBBP4	↑	↑
TGFβI	↓	↓

↑: mean expression was greater then 1.5
↓: mean expression was less than 1.5

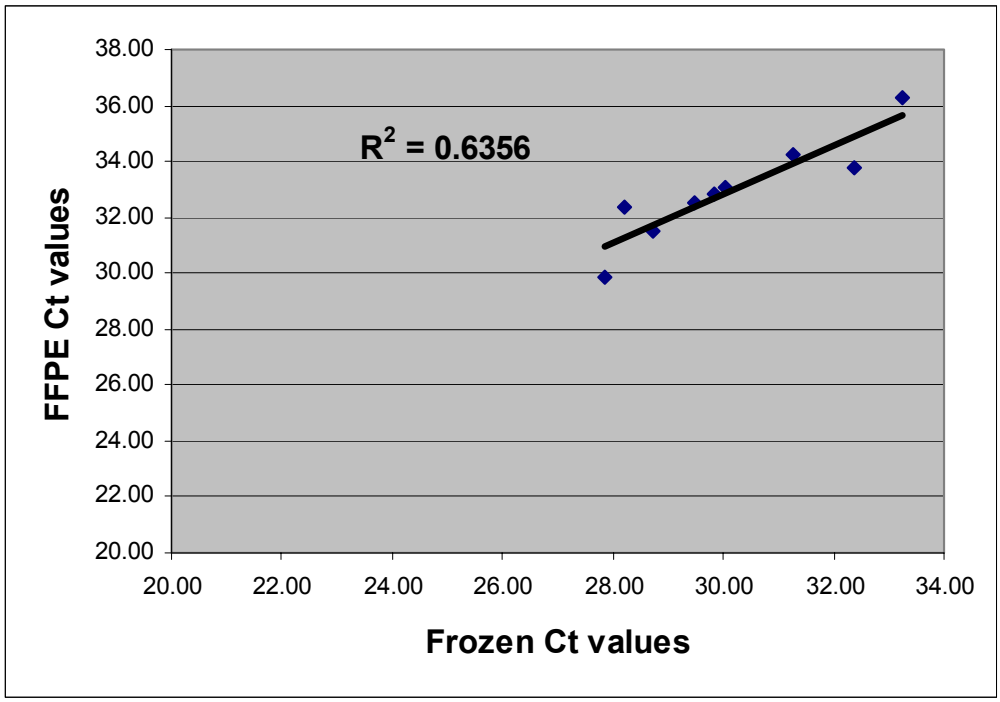
3.6 Target gene expression in FFPE tumour samples

Analysis of target genes using RNA from snap frozen tumour tissue was restricted by the limited availability of tissue, particularly when considering cancers from ≤35 years, which are rarer. The ability to analyse RNA from FFPE tumours would enable many more cases to be considered. Initially 9 FFPE cases were examined for which data were available for the target genes from snap frozen tissue (see Table 3.12). A further 14 FFPE (≤35 years, and <35 years) were also examined.

For each case, ten 4 μM thick sections were cut and RNA was extracted (see section 3.2.1.1). As with the snap frozen tissues, three micrograms of total RNA was converted to cDNA using reverse transcriptase as outlined in sections 2.2.3.1 and 2.2.7.1. The cDNA was then examined for the expression of the nine target genes using SYBR Green real-time quantitative PCR (see section 2.2.8). Each experiment was run in triplicate and all results were represented as relative expression (target gene expression relative to the average of the 3 housekeeping genes (Target/Housekeeping gene). Statistical analysis using a 2-tailed independent t-test was performed to determine whether or not there were any significant differences in the amount of target gene in each the group.

The housekeeping genes (HPRT1, TFRC, PGBD) expression profiles of the FFPE tissues had a weaker correlation with the profile generated from matched snap frozen specimens (R^2 = 0.635) (see Figure 3.38). The differences in the raw Ct values between the specimen types ranged from 1.39 to 4.15. Although the RT-qPCR detected all nine target genes, and housekeeping genes in all FFPE tumour samples, there appeared to be great variation in the amount of each gene in matched snap frozen and FFPE samples.

Figure 3.38 Correlation between the Housekeeping gene profiles in matched snap frozen and FFPE tissues.



Mean expression of the 3 housekeeping genes in 9 matched (X-axis: snap frozen and Y-axis: FFPE) tumour samples.

Table 3.12 Patient details of FFPE tissues

RW No	Snap Frozen
2195	Y
2215	Y
2200	Y
2019	Y
2201	
2204	Y
2196	
2137	
2105	
2220	
2140	Y
2185	Y
2093	Y
2182	Y
2070	
2149	
2183	
2047	
2139	
2095	
2096	
2074	
2076	

Y: data from snap frozen specimens also available.

3.6.1 Comparison of frozen and FFPE

When relative expression levels of each target gene were compared for frozen and FFPE tissue there were striking differences. Overall, there was a higher level of expression for RNA from frozen samples than FFPE samples (see Table 3.13). For RARRES3, all snap frozen specimens had higher relative expression levels compared to the FFPE tissues. For granulin, all but one snap frozen specimen showed higher relative expression levels compared to the matched FFPE tissues. For example, for AKAP1, the FFPE tissue sample of case number 2140 had a relative expression level of 8.7 whereas the same case saw a relative expression value of 24.04 in the snap frozen specimen. Similarly, case number 2182 was found to have contrasting relative expression levels in the FFPE and snap frozen specimens with values of 3.29 and 0.63 respectively. There were some discrepancies that are difficult to explain eg. case # 2019 for AKAP1. Also, for RBBP4, four of the FFPE samples had higher levels of expression. Since overall CT values were higher for FFPE than frozen tissue for the same cases, it was concluded that snap frozen tissue was more likely to reflect accurate results.

Table 3.13 Summary of relative expression in matched snap frozen and FFPE tumour tissue

	FFPE	Frozen	FFPE	Frozen	FFPE	Frozen	FFPE	Frozen
Case number	**AKAP1 Relative expression**		**C/EBP α Relative expression**		**Granulin Relative expression**		**RARRES3 Relative expression**	
2140	8.70	**24.04**	**3.45**	0.08	1.66	**7.69**	0.24	**1.81**
2185	**16.76**	3.08	0.36	**0.81**	1.68	**8.25**	0.15	**3.02**
2182	**3.29**	0.63	**0.44**	0.09	**3.47**	1.25	0.23	**2.30**
2093	3.64	**28.20**	0.32	**1.99**	0.58	**15.20**	0.13	**10.05**
2215	4.31	**9.28**	1.04	**3.75**	1.66	**8.97**	0.84	**14.37**
2204	5.66	**20.73**	**1.78**	0.16	2.47	**7.06**	0.10	**24.29**
2200	5.32	**5.94**	0.29	**1.27**	1.42	**5.82**	0.22	**17.94**
2195	0.61	**10.09**	**0.46**	0.05	0.30	**2.84**	0.23	**14.62**
2019	**49.10**	1.79	**1.97**	0.05	4.16	**11.38**	0.27	**3.52**
	FFPE	**Frozen**		**FFPE**	**Frozen**		**FFPE**	**Frozen**
Case number	**APRIL Relative expression**		**DDB2 Relative expression**		**NCOA3 Relative expression**		**RBBP4 Relative expression**	
2140	0.15	**12.03**	1.21	**46.37**	**1.22**	0.76	**5.07**	3.41
2185	0.40	**3.09**	0.47	**30.25**	0.16	**3.20**	**5.04**	4.93
2182	**0.88**	0.57	0.05	**0.77**	0.26	0.13	**6.85**	3.88
2093	0.13	**9.74**	0.38	**17.83**	**0.29**	0.19	1.84	**9.36**
2215	2.19	**2.91**	**4.79**	1.77	5.21	**6.54**	6.37	**18.02**
2204	**2.34**	0.42	1.98	**3.90**	0.16	**3.27**	5.82	**48.53**
2200	0.40	**5.43**	0.46	**0.66**	0.22	**0.44**	1.19	**14.52**
2195	0.11	**8.16**	0.20	**1.48**	0.07	**4.23**	0.77	**7.96**
2019	0.99	**4.75**	**4.11**	0.22	0.34	**1.79**	**2.81**	0.70

Case number	**TGFβI Relative expression**	
2140	0.09	**6.748**
2185	0.15	**3.156**
2182	0.06	0.286
2093	0.28	**2.495**
2215	**0.75**	0.461
2204	0.20	**1.086**
2200	0.10	**0.218**
2195	0.08	**0.633**
2019	**1.10**	0.429

Relative expression levels of all 9 targets genes in matched fixed and snap frozen tumour specimens. Values in bold represent the higher relative expression in matched samples.

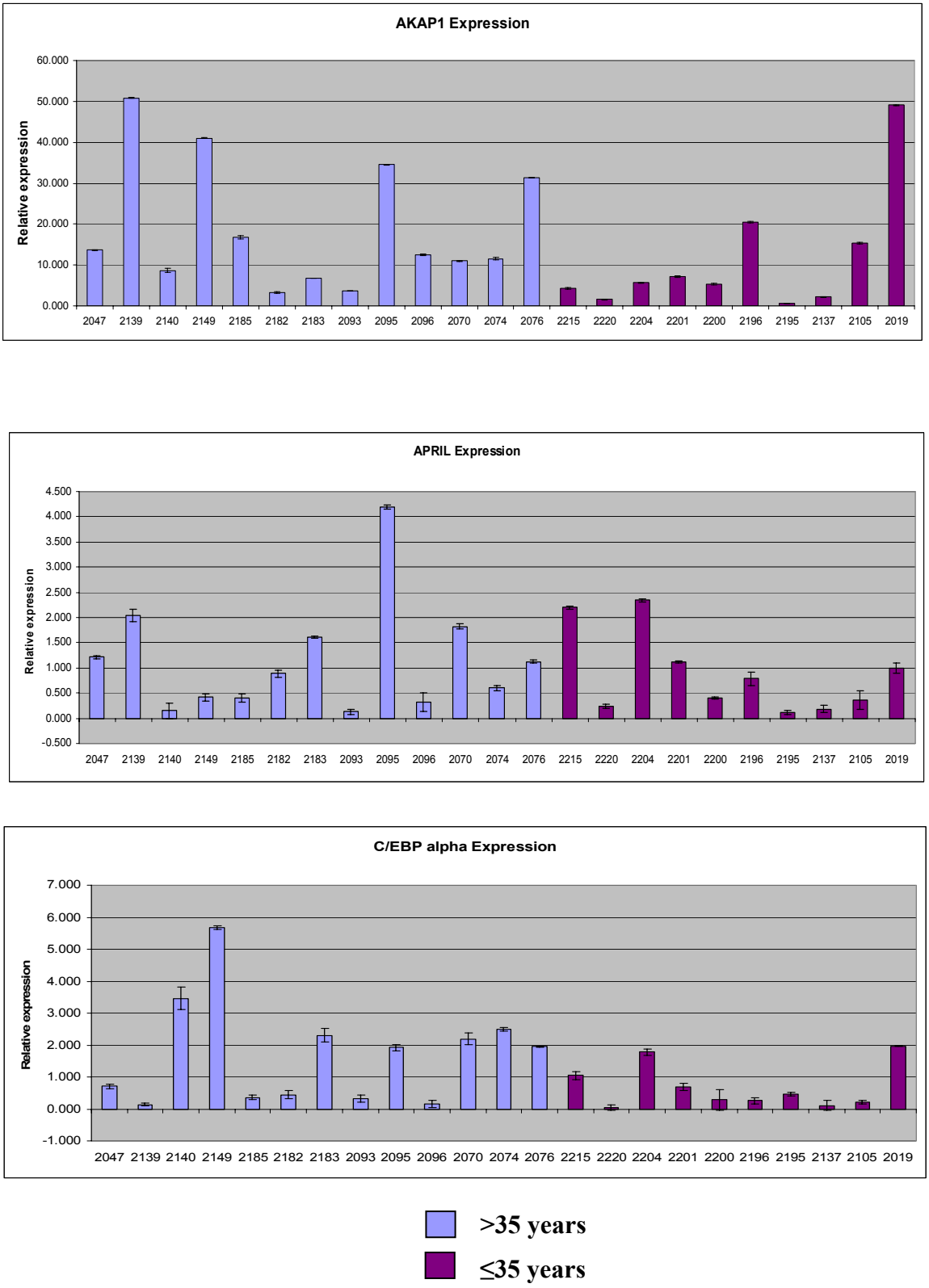
3.6.2 Gene expression in FFPE tissues

RT-qPCR in FFPE breast tumours showed a predominantly high relative expression of AKAP1 (see Figure 3.39), which supports the microarray and the RT-qPCR analysis of snap frozen tissues; but like the snap frozen analyses, great variation in the relative expression levels was found. Relative expression levels were above 2.00 with the exception of sample # 2195, a younger breast tumour sample. There was no difference in the amount of AKAP1 expression between the two groups ($p=0.07$).

Analysis of APRIL expression found relative expression levels to be lower than 2.00 in all FFPE tumour samples, with the exception of two younger cases (#2215, and 2204), and two older cases (#2139, and 2095) (see Figure 3.39). Although APRIL relative expression was lower in FFPE tissues compared to frozen samples, as for the frozen samples, no difference in the expression between each age groups was observed ($p=0.982$).

C/EBP alpha expression was predominantly low across all FFPE tumour samples cases with the exception of 5 cases in the >35 years old group (see Figure 3.39), which is in agreement with the data from frozen tumours. Although the C/EBP alpha expression appeared higher in the >35 years age group compared to the <35 years old age group, this difference was not statistical significant ($p=0.068$).

Figure 3.39 AKAP1, APRIL, and C/EBP alpha mRNA expression in FFPE tissues



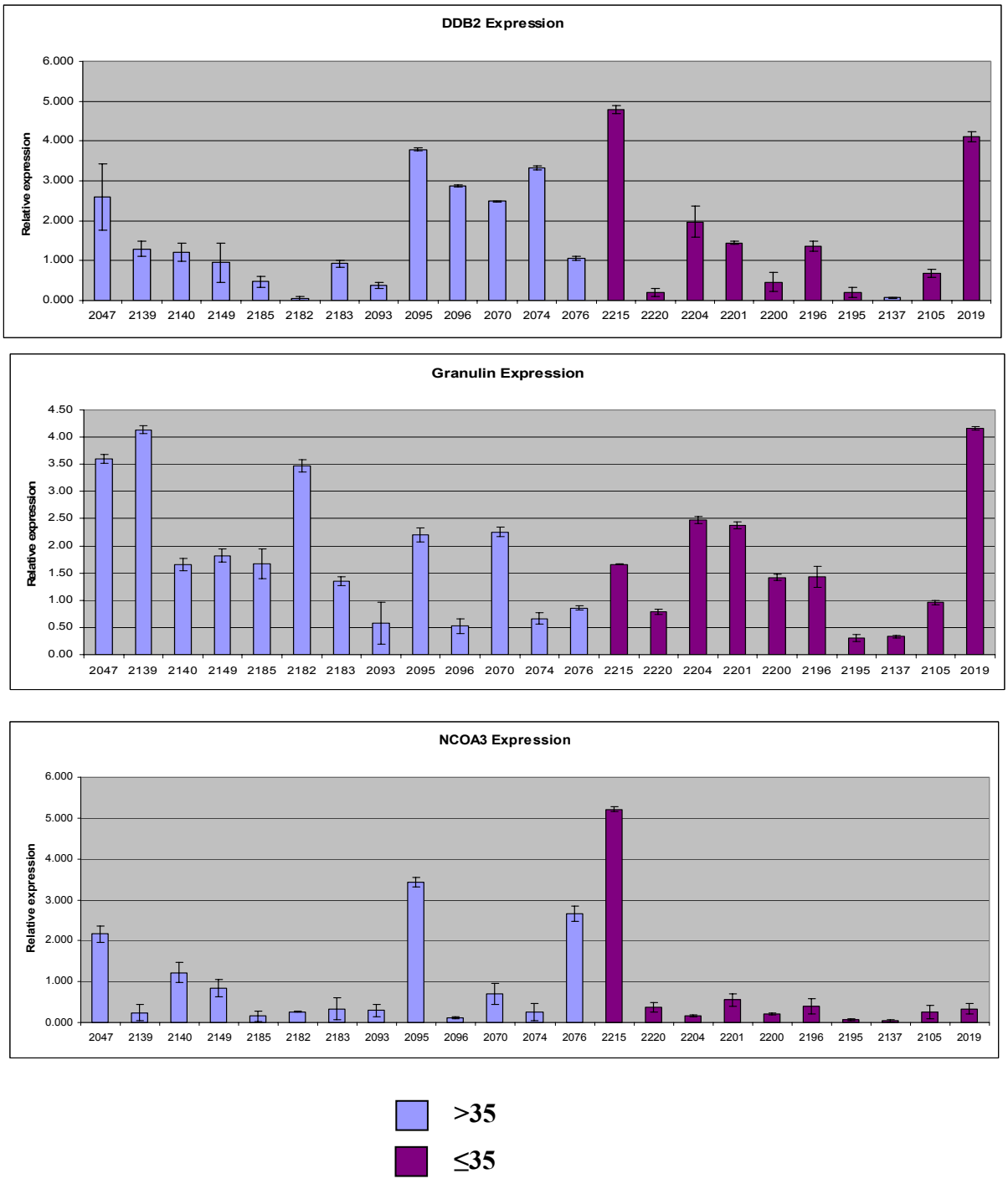
Error bars reflect standard error of the mean (SEM).

As with the frozen tumours, RT-qPCR analysis of DDB2 saw varied expression among all FFPE tumour samples cases (relative expression ranged from 0.05 in an older tumour case (#2182) to 4.71 for a younger tumour case (#2215) (see Figure 3.40). Relative expression was below 2.00 in 8/13 tumour from women >35 years, and in 8/10 tumours from women ≤35 years. No difference was observed in the expression of DDB2 between the two age groups ($p= 0.246$).

The amount of granulin expressed in FFPE tumour tissues varied greatly (see Figure 3.40). Relative expression levels on the whole remained below 2.00, which differ from the results from frozen samples. No difference in expression between the two age groups was observed ($p=0.968$).

In the case of NCOA3, (a gene that had shown differential expression between age groups in snap frozen tumours), the results from the FFPE tissues analyses saw no obvious differences in the relative expression levels between the two age groups in the 2 genes ($p=0.194$) (see Figure 3.40). Relative expression levels were below 2.00 in all FFPE with the exception of 3 cases >35 years old, and one case ≤35 years old.

Figure 3.40 DDB2, Granulin, and NCOA3 mRNA expression in FFPE tissues



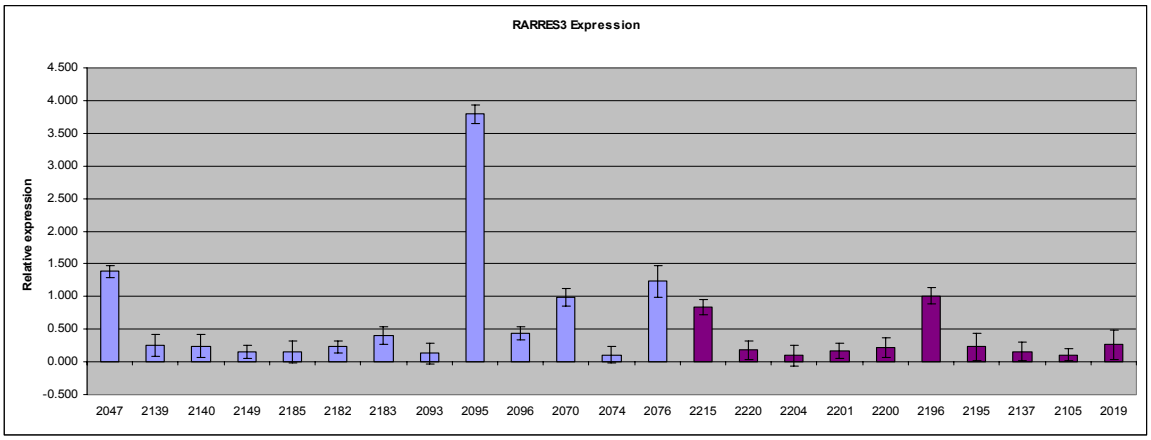
Error bars reflect standard error of the mean (SEM).

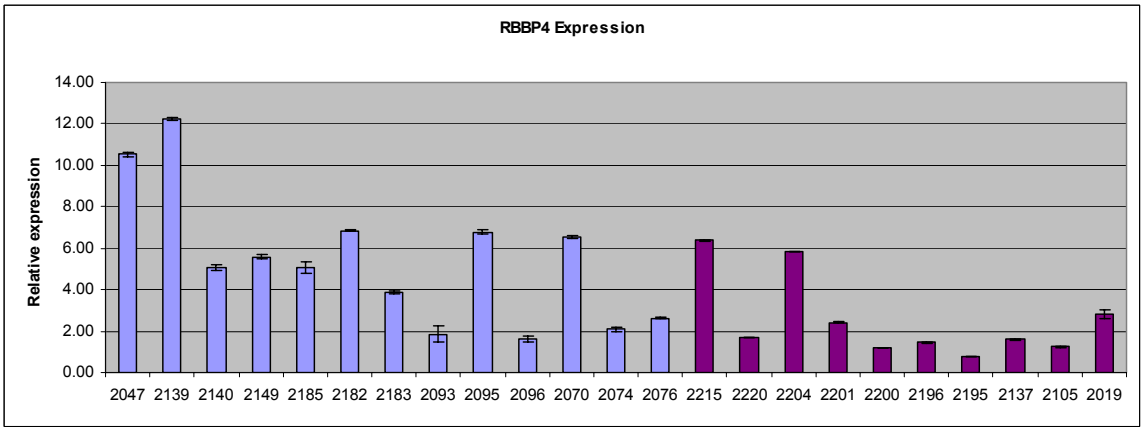
RARRES3, another gene found to have differential expression among tumours from women >35 years and those ≤35 years following qPCR analysis of snap frozen tumour samples, showed no difference in its expression between age groups in FFPE tissues (p=0.217) (see Figure 3.41). With the exception of one case (>35 years), all samples had relative expression levels below 2.00.

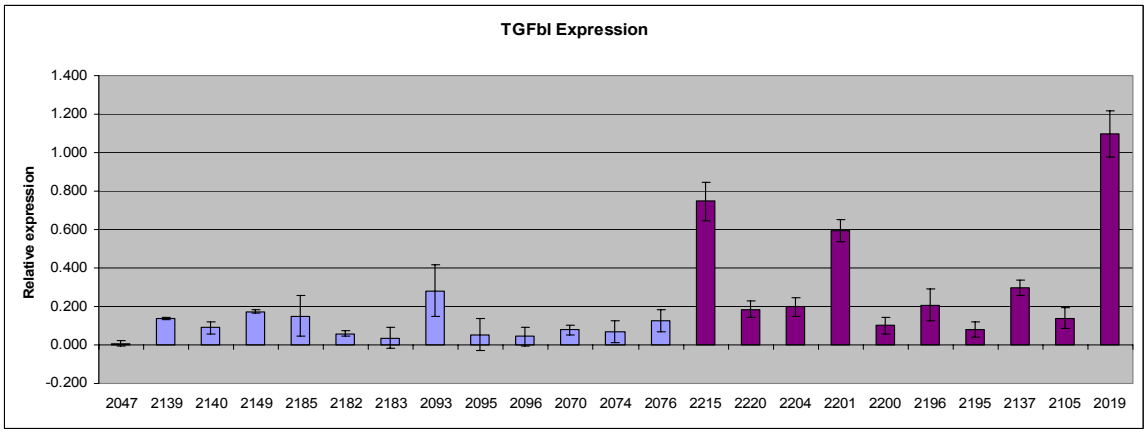
The results of the qPCR analysis of RBBP4 in FFPE tissues saw predominantly high expression (relative expression > 2.00) in both tumour age groups, which is in agreement with the results from frozen tissues (see Figure 3.41). While the cases >35 years appeared to have a higher expression compared to those ≤35 years, this difference was not significant (p=0.524).

In all but one FFPE tumour samples the relative expression of TGFβI was below 1.00 (see Figure 3.41). The expression in ≤35 group was in general higher than that of the >35 year old cases (p=0.006). This is in contrast to the snap frozen samples, where tumours from women >35 years appeared to have higher levels of expression.

Figure 3.41 RARRES3, RBBP4, and TGFβI mRNA expression in FFPE tissues







Error bars reflect standard error of the mean (SEM).

3.7 Expression in tumour versus normal: data comparisons

For the freshly frozen tumours analysis of variance (ANOVA) was used to determine whether the relative expression of a target gene was statistically different between the 2 age groups, and in comparison to the organoids. These comparisons are presented in Figure 3.42. One-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test was used since the organoids were incorporated in the analysis to determine whether the expression nine genes was altered in normal and cancerous breast for either age group.

Only NCOA3 and RARRES3 were found to have a significantly higher expression in breast cancers in younger women compared to older women, and to the normal organoids (for NCOA3 the P values were 0.001, 0.024, and for RARRES the P values were 0.002 and 0.043 respectively).
The analysis of AKAP1, RBBP4 or APRIL expression in organoids and tumour specimens saw no significant difference between any of the groupings.

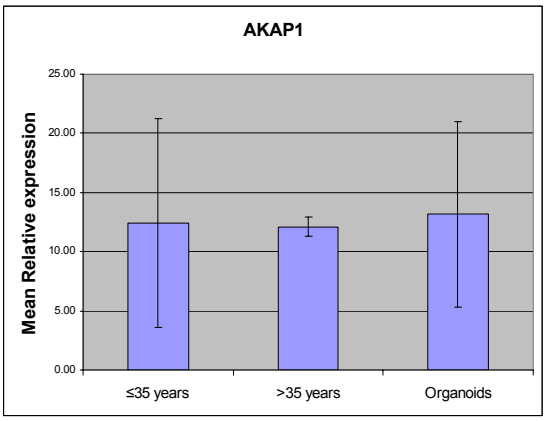
C/EBP alpha expression was very low in all tumour specimens, whereas the organoids showed higher levels of mRNA expression. The expression levels in the two tumour groups did not differ from each other, however they were significantly different to the organoids (P= 0.001, and P=0.009), suggesting an overall down regulation in breast cancers.

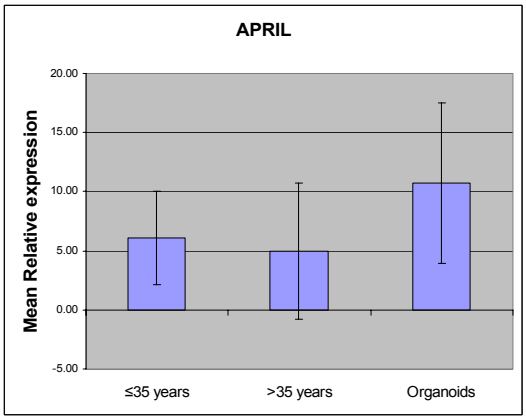
DDB2 expression profiles were comparable in the two age groups in the snap frozen cases. Organoids from reduction mammoplasty samples had a higher expression compared to tumour in women ≤35 years old and >35 years old, although this difference was only statistically significant in ≤35 years age group (P= 0.001). The level in cancers from women ≤35 years was low with tight standard deviation, whereas the standard deviation for cancers from women >35 years was wide, and hence there was no statistical difference between the two groups, despite the latter being higher.

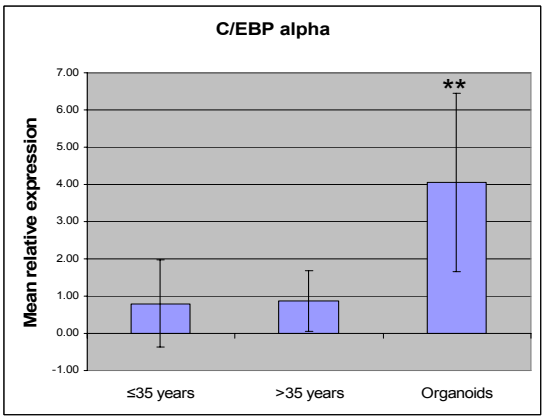
Analysis of granulin expression saw a significantly higher level of expression in tumours from women ≤ 35 years compared to the normal organoids (P=0.018). No difference was found between tumours in younger and older women.

TGFβI expression was found to be significantly lower in both tumour age groups compared to the organoids samples (P= 0.001 and 0.026).

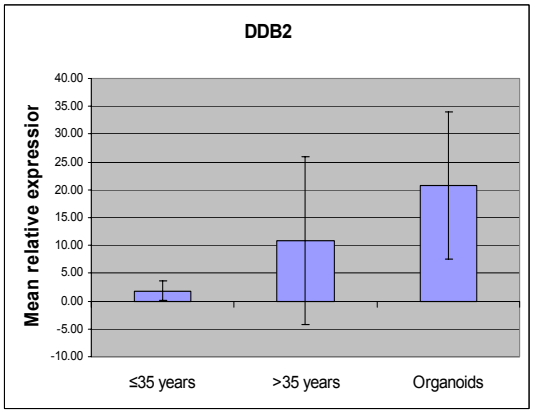
Figure 3.42 Summary data comparisons



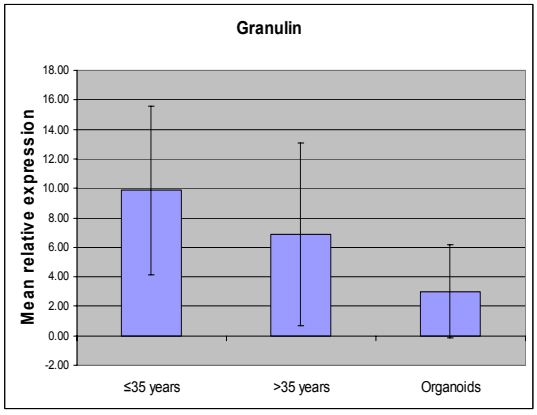




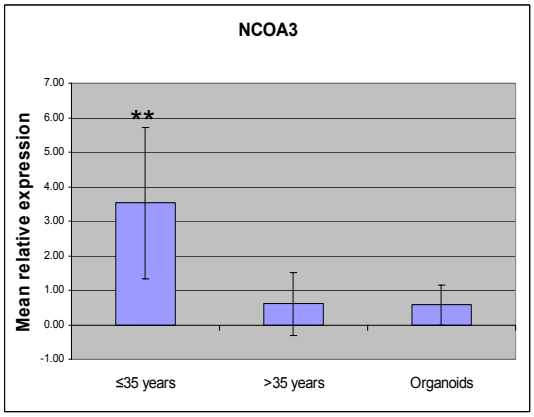
≤ 35 v organoids P= 0.001
> 35 v organoids P=0.009



≤ 35 v organoids P= 0.002

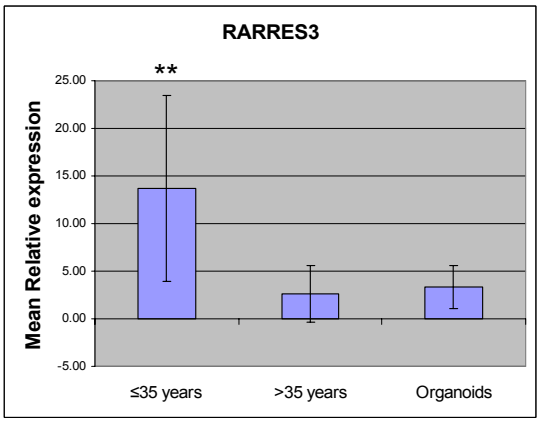


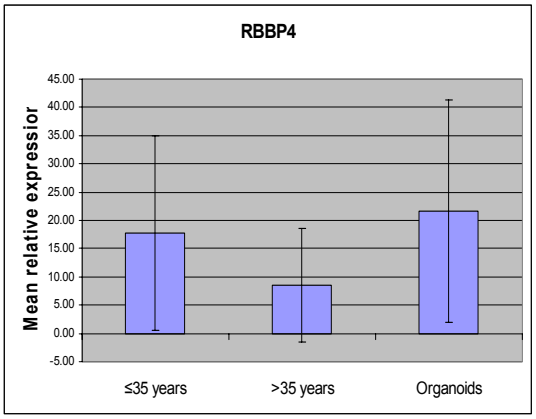
≤ 35 v organoids P= 0.018



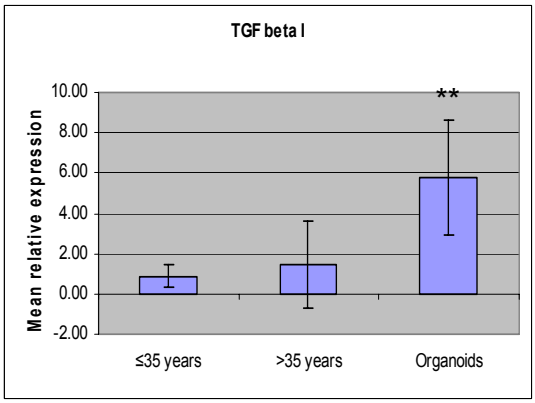
≤ 35 v >35 P = 0.001
≤ 35 v organoids P= 0.024

Figure 3.42 continued.





≤ 35 v >35 P = 0.002
≤ 35 v organoids P= 0.043



≤ 35/ >35 v Organoids P= 0.001 and 0.026

Each graph shows the mean relative expression of each target gene in tumour samples stratified by age, as well as in organoids samples. Error bars represent standard deviation from the mean. ** Statistically significant.

Of the nine target genes investigated by qPCR, five showed differences between normal and cancers ≤35, or between breast cancer ≤35 and those >35. NCOA3 and RARRES3 showed differential expression between younger and older cases, with NCOA3 and RARRES3 displaying elevated levels of mRNA in breast cancers ≤35 years. DDB2 and C-EBPα showed a reduced level of mRNA expression in both younger and older cases compared to the normal breast tissue but for DDB2 this difference was statistically significant only in the younger group. TGFβI expression was significantly lower in both tumour age groups compared to the normal organoids. Granulin showed elevated mRNA expression in younger breast tumours compared to the normal organoids. See Table 3.14 for a summary of P-values.

Table 3.14 Summary of P-values for target gene expression in snap frozen tissues

Target Gene	≤35 years v >35 years	≤35 years v Organoids
AKAP1	0.557	1.00
APRIL	0.823	1.00
C/EBP alpha	0.941	**0.001**
DDB2	0.355	**0.002**
Granulin	0.420	**0.018**
NCOA3	**0.001**	**0.024**
RARRES3	**0.002**	**0.043**
RBBP4	0.446	1.00
TGFβI	0.566	**0.026**

ANOVA was carried out to verify whether target gene expression levels were statistically significantly different between tumours (≤35 years v >35 years), and between tumours from women in these age groups and normal organoids. Values in bold represent those that were significantly different.

3.8 Protein analysis of NCOA3 and RARRES3

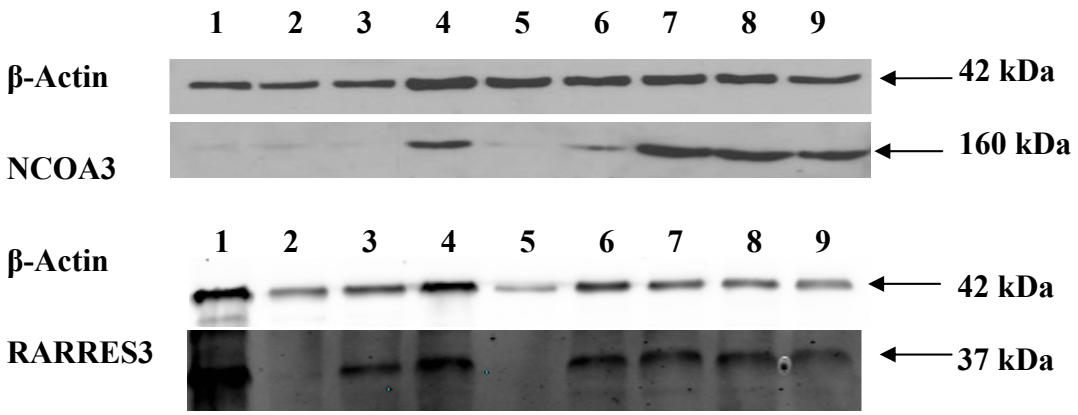
The hypothesis tested in this thesis is that breast cancers occurring in younger women (≤35 years) differ from those from older women. Of the nine candidate genes assessed by qPCR analysis, NCOA3 and RARRES3 showed significant difference in their mRNA expression between tumours from women ≤35 years old and >35 years old as well as to normal breast tissue. To investigate the mRNA expression reflected changes in protein levels, protein expression analysis was carried out. A product search for antibodies was carried out; a monoclonal NCOA3 antibody which had applications for both western blotting and IHC was identified, but for RARRES3, only a polyclonal antiserum for use in western blotting was found. Therefore, analysis of NCOA3 was undertaken by (1) western blotting in breast cell lines and organoids and, (2) immunohistochemistry (IHC) on a larger panel of breast tumours from both age groups, as well as on a panel of normal breast specimens taken from reduction mammoplasties. RARRES3 was analysed by western blotting of breast cell lines and organoids.

3.8.1 Western blotting analysis of NCOA3 and RARRES3

Western blotting for NCOA3 (160kDa) and RARRES3 (37kDa) proteins was carried out on the same 6 breast cell lines used for RT-qPCR (HBL-100, MDA-MB-231, MDA-MB-468, MCF-7, ZR-75-1, and T47-D), and on three normal breast organoids samples (1978, 1960, and 1967). Sample 1978 had been previously used in the mRNA analysis. None of the other 8 organoids samples could be used for protein studies due to limited availability of material. Twenty-five micrograms of cell lysate were separated on an 8% SDS-PAGE gel (NCOA3), and a 12% SDS-PAGE gel (RARRES3), transferred to nitrocellulose membranes, and immunoblotted with anti-NCOA3, and anti-RARRES3 antibodies (see section 2.2.9). β-Actin was used as a loading control.

Strong NCOA3 protein expression was found in the oestrogen positive cell lines MCF-7, ZR-75-1, and T47-D, with moderate expression in HBL-100. A weak band was seen for MDA-MB-468, but there was no detectable protein in the organoids and MDA-MB-231 cells (see Figure 3.43). The RARRES3 protein was detected in 2 out of 3 organoids samples (1978 and 1967) and 4 breast cell lines (HBL-100, MDA-MB-468, MCF-7, and ZR-75-1).

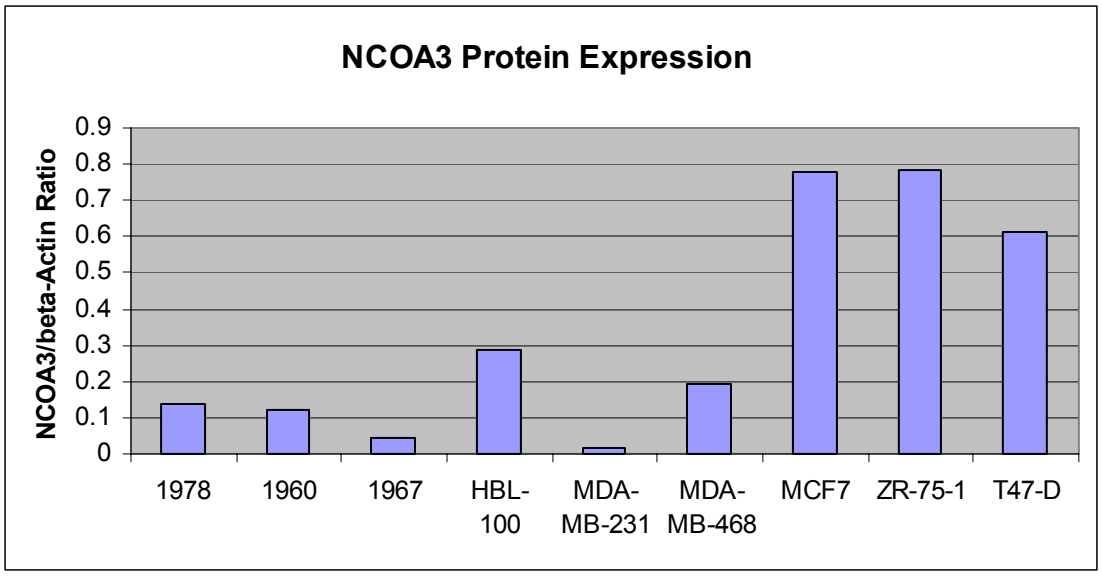
Figure 3.43 Protein expression in cell lines and organoids



Lanes: 1, RM 1978 ; 2, RM 1960; 3, RM 1967 ; 4, HBL-100; 5, MDA-MB-231; 6, MDA-MB-468; 7, MCF-7; 8, ZR-75-1; 9, T-47-D.

Densitometric analyses of NCOA3 protein expression in relation to the β-Actin provided a semi-quantitative representation of the expression levels across all samples (see Figure 3.44). This confirmed that there were higher levels in the ER-positive breast cell cancer lines, which supports the expression seen at the mRNA level.

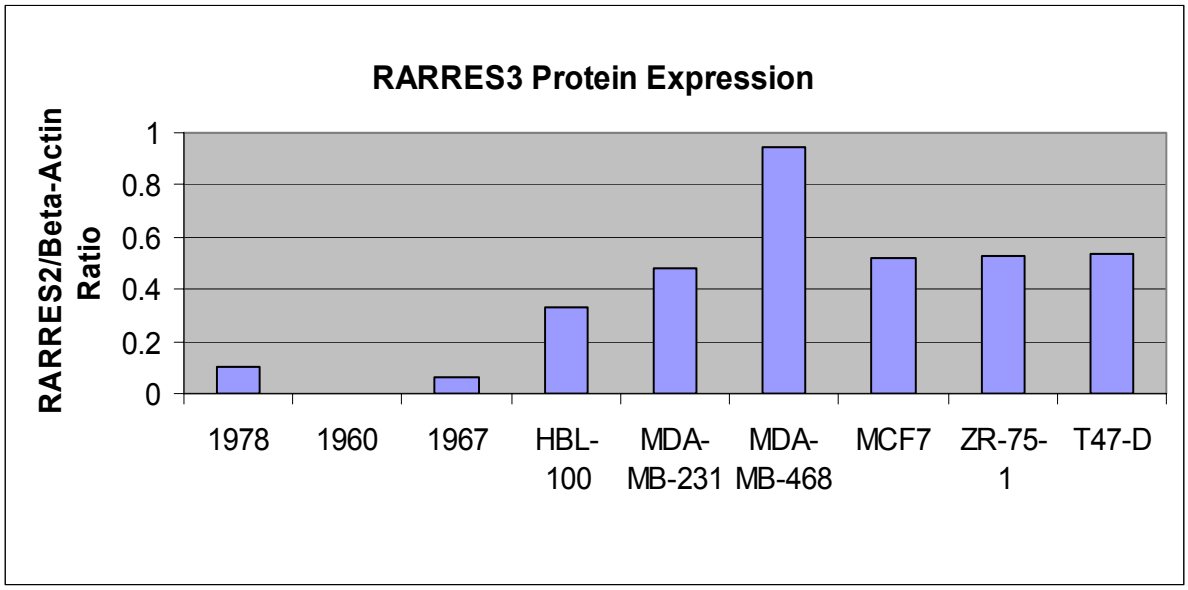
Figure 3.44 Densitometric analysis of NCOA3 protein expression in breast cell lines and organoids



Normalisation of NCOA3 to β-Actin expression

Normalisation of RARRES3 to beta-Actin using densitometry showed high relative amounts of RARRES3 in the organoids samples, HBL-100, MDA-MB-468, ZR-75-1, with lower levels in MCF-7, T47-D, and MDA-MB-231 cells (see Figure 3.45).

Figure 3.45 Densitometric analysis of RARRES3 protein expression in breast cell lines and organoids



Normalisation of RARRES3 to β-Actin

For NCOA3 both mRNA and protein expression follow the same trend with relatively higher levels in the ER-positive cell lines compared to the ER-negative MDA-MB-231 and MDA-MB-468 cells, non tumourigenic HBL-100 and the normal organoids. In the case of RARRES3, high levels of mRNA and protein were found in organoids sample 1978 and the breast cancer cell line MDA-MB-468. However, in contrast to mRNA findings relatively higher levels of the RARRES3 protein were found in HBL-100, MCF-7, and ZR-75-1.

3.8.2 Immunohistochemical analysis of NCOA3

3.8.2.1 Optimisation

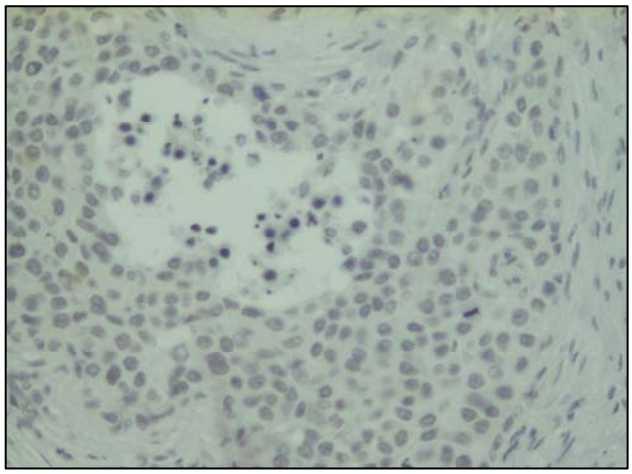
The optimisation of the NCOA3 antibody was achieved by a variety of modifications: 1) adjusting the dilution factor used for the primary antibody, 2) varying the temperature and duration of antigen retrieval pressure cooker method, and 3) testing an alternative antigen retrieval method (micro-waving). For a summary of the optimisation methods see section 2.2.10. To optimise the antibody, a tumour case (#2101) that showed strong HER-2 expression was chosen, as previous studies have found a correlation between the expression of NCO3 and HER-2 (Bouras *et al.*, 2001; Kirkegaard *et al.*, 2007; Osborne *et al.*, 2003). The optimal conditions for the IHC were deemed those which yielded the most intense staining with least background staining, with no staining in the negative control. Example results from the optimisation process can be seen in Figure 3.46 with a summary of the pressure cooking parameters in Table 3.15. Antigen retrieval using the pressure cooker methods produced better results than the microwave (more intense staining with less background staining) (photo A, versus B and C). In photo A, none of the cells are stained, whereas in photo B there is weak reactivity, and in photo C there is substantial nuclear staining with weaker cytoplasmic staining. Therefore, pressure cooker at 125 degrees for 45 seconds, with a primary antibody dilution 1:40 was selected as the optimal conditions for the assessment of NCAO3 in tumour tissues (see photo C).

Table 3.15 Summary of antigen retrieval parameters

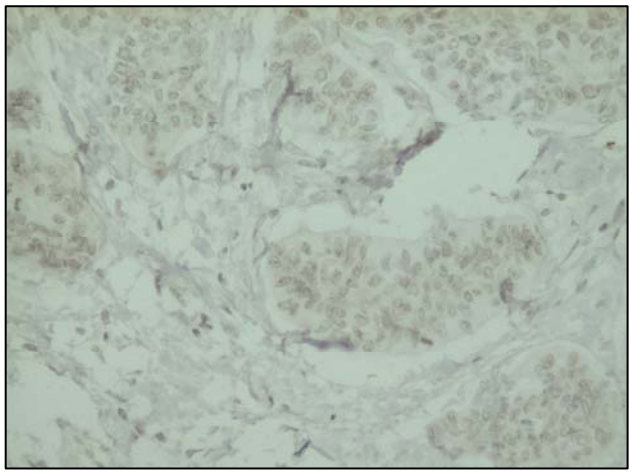
Pressure Cooker		Antibody dilutions
Temperature	**Time Duration**	
120º	30/45/60 seconds	1:20/40/100/200
123º	30/45/60 seconds	1:20/40/100/200
124º	30/45/60 seconds	1:20/40/100/200
125º	30/45/60 seconds	1:20/40/100/200

Figure 3.46 Summary of optimisation process for NCOA3 IHC

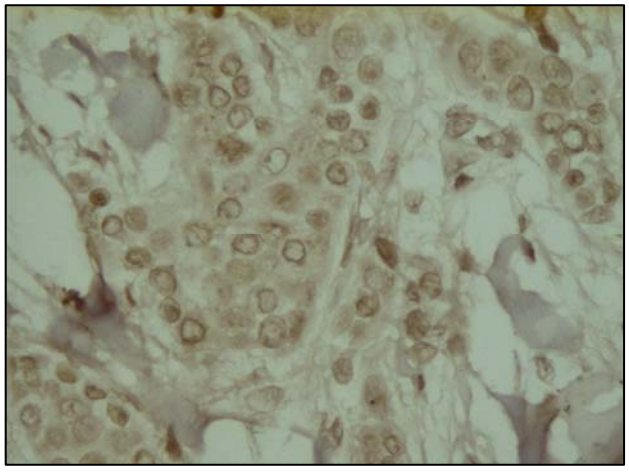
A.



B.



C.



A: Ab diluted 1:40 and antigen retrieval carried out using a microwave at full power for 20 minutes is represented; B: antigen retrieval carried out in a pressure cooker at 123° for 60 seconds, Ab diluted 1:40; C: antigen retrieval carried out in a pressure cooker at 125° for 45 seconds, Ab 1:40.

3.8.2.2 Normal breast

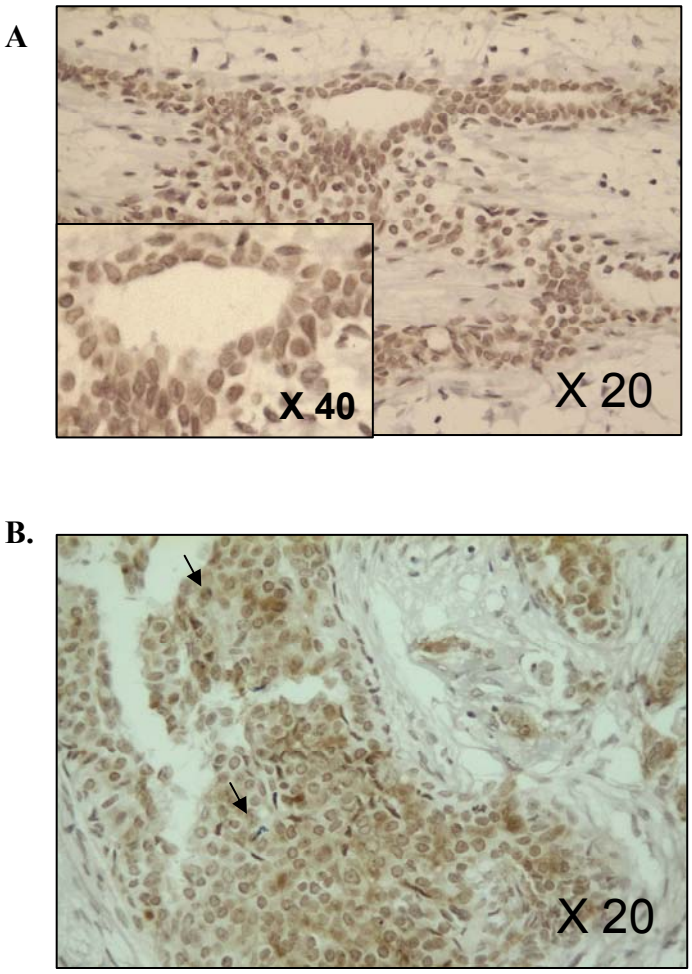
In total 12 samples of normal breast tissue were examined for NCOA3 protein expression. Each tissue was run alongside a negative control of the same specimen where primary antibody was replaced with normal rabbit serum at the same dilution. In addition, one case 2101 was included in every experimental run as a positive control for inter-assay reproducibility. Staining extent was graded as the percentage of positively stained nuclei, and categorised as negative (<1% staining), low (1-5% staining), moderate (5-50% staining), and high (>50% staining). Nuclear staining was observed in luminal and myoepithelial cells of four of twelve normal breast samples (see Figure 3.47, Table 3.16). In one case NCOA3 was detected in >50% of the cells. Three other samples had evidence of staining but only in a small percentage of cells. The rest were negative. There was no correlation of protein expression with age.

Table 3.16 Summary of NCOA3 IHC for 12 normal breast tissues

Samples Number	Age	Staining
0706	18	1-5%
0907	20	Neg
6405	20	>50%
1206	21	Neg
0606	21	Neg
0806	28	Neg
6305	29	1-5%
1006	32	Neg
0607	37	Neg
0807	43	Neg
0407	47	5-50%
0506	54	Neg
Mean age	30.8	

Age: age of donor at time of reduction mammoplasty

Figure 3.47 NCOA3 IHC for normal breast tissues.



A: Case 6405, strong staining; B: Case 2101 (positive control). Arrows point to cells that show strong nuclear staining.

3.8.2.3 Breast cancers

In total, 56 breast tumour samples (20 ≤35 years, 15 36-49 years, and 21 ≥50 years) (see Table 3.17) were examined for NCOA3 protein expression. Each case was run alongside a negative control of the same specimen where primary antibody was replaced with normal rabbit serum at the same dilution. Case 2102 was included in every experimental run to control for inter-assay reproducibility. As for normal breast tissue, staining extent was graded as the percentage of positively stained nuclei, and categorised as negative (<1% staining), low (1-5% staining), moderate (5-50% staining), and high (>50% staining). Cytoplasmic staining was recorded as being present or not.

Table 3.17 Summary of clinicopatholigical data from all age groups for 56 breast carcinomas
IDC: Infiltrating ductal carcinoma; ILC: infiltrating lobular carcinoma; Pos: positive; Neg: negative.

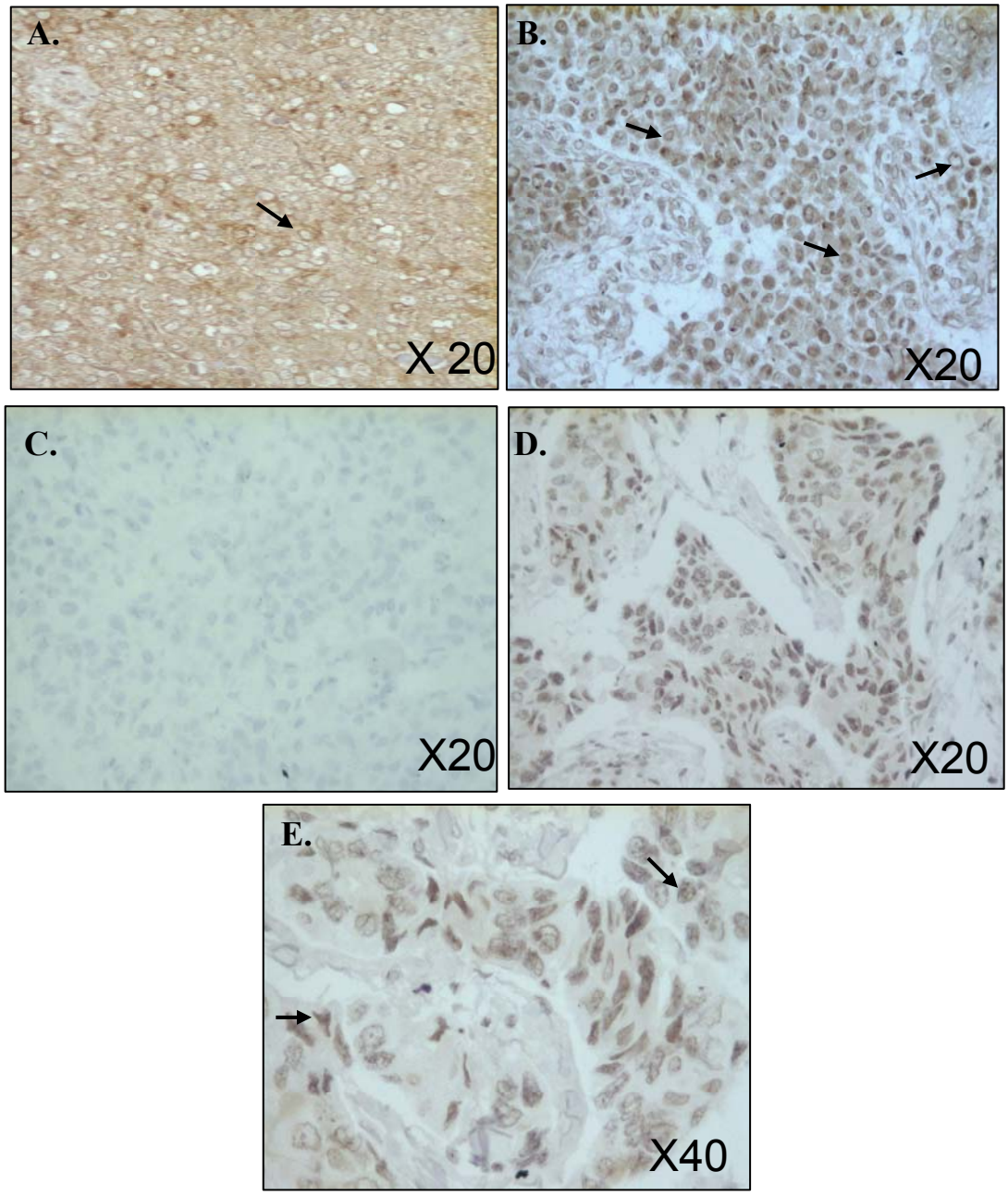
Age Range	≤35	36-49	≥50
N	20	15	21
Mean Age	31.7	44.5	58.4
Type			
IDC	20	15	20
ILC	0	0	1
Grade			
I	0	0	2
II	5	3	8
III	15	12	11
Node status			
Pos	9	8	11
Neg	11	7	10
ER status			
Pos	12	10	16
Neg	7	5	4
PgR status			
Pos	13	9	15
Neg	6	6	5

In the 20 breast cancers from younger women, 6/20 (30%) showed NCOA3 staining. One case had low nuclear staining, one showed moderate staining, and four showed high staining (see Figure 3.48). One case with high staining showed both nuclear and cytoplasmic staining (see Figure 3.48 B).

For the 15 breast cancer samples from women 36-49 years, 3 showed high staining, 2 had moderate staining, 3 low staining, and 7 were negative, making 8/15 positive (53.3%). One of the tumours with high staining had both nuclear and cytoplasmic staining.

In the 21 tumour samples from women ≥50 years, 4 had high staining, 5 had moderate staining, 3 had low staining, and 9 were negative, making 12/21 positive (57.1%). Again, one of the tumours in this age group with high staining had both nuclear and cytoplasmic staining present.

Figure 3.48 NCOA3 IHC on breast tumour samples



Panels A shows high cytoplasmic staining (Case #2111); panels B shows high nuclear and cytoplasmic staining in a cancer case ≤ 35 years #2204 (C: negative control); panels D&E show high nuclear staining in a breast cancer case ≤ 35 years #1857. Arrows point to areas of strong staining.

Table 3.18 shows a summary of NCOA3 immunohistochemistry results and a comparison with clinical prognostic factors such grade, and hormone status. A Pearson Chi-squared test was performed to determine whether NCOA3 expression was correlated with age, grade, and hormone status. Unfortunately, there was insufficient information regarding the HER-2 status of the samples to explore whether there was a correlation between its expression and that of NCOA3. No significant correlation was found between NCOA3 expression and age (≤35 years, 36-49 years, and ≥50 years), grade, and ER/PgR status. When the cases were stratified into (≤35 years and >35 years), a higher number of breast tumours from women >35 years had positive NCOA3 staining (19 versus 9), however this relationship was only approaching significance (p=0.066).

Table 3.18 Relationship of NCAO3 staining to age, grade, and hormone status

	NCOA3 staining		
	Negative	**Positive**	**P-value**
Age			0.855
≤35 years	11	9	
36-49 years	7	8	
≥50	10	11	
>35*	17	19	0.066
Normal	8	4	
Grade			0.934
I	1	1	
II	8	8	
III	20	18	
ER status			0.127
Pos	18	21	
Neg	11	5	
PgR status			0.234
Pos	17	20	
Neg	11	6	

P value: Pearson's chi-squared statistical test
*tumour cases stratified into those ≤35 years and >35 years.
Normal: normal breast tissue taken from reduction mammoplasties

3.8.3 Correlation of IHC with RT-qPCR results

It was possible to compare NCOA3 immunohistochemistry results with RT-qPCR data for 23 cases (see Table 3.19). Eight cases showed moderate or high NCOA3 expression at the protein level, but only 3 out of these showed levels of NCOA3 mRNA expression greater than that of the mean of 3 housekeeping genes. For example, case number 2204 had high NCOA3 protein staining, and had a relative level of mRNA expression of 3.27. The remaining 5 cases that showed moderate or high NCOA3 staining in IHC all had lower mRNA levels compared to the housekeeping genes. Incidentally, the information for mRNA expression for cases #2137 and 2105 was taken from RT-qPCR carried out on FFPE tissues. Of the 2 cases that had low NCOA3 protein expression (2140 and 2022), the matching relative mRNA levels were lower than those of the housekeeping genes. For example, 2022 had a relative mRNA level of 0.14. However, the FFPE specimen had a relative expression of 1.22. Two cases that had high mRNA relative expression lacked NCOA3 protein by IHC (2157 and 2209).

Table 3.19 Summary of NCOA3 expression at the mRNA and protein levels

Case No.	Age	Grade	ER	NCOA3 protein	NCOA3 mRNA relative expression	
					Frozens	FFPE
2157	19	III	Pos	Neg	4.00	NA
2144	30	III	Neg	Neg	0.90	NA
2019	31	III	Pos	>50%	1.79	0.336
2201	32	III	Pos	Neg	NA	0.55
2200	34	III	Neg	Neg	0.44	0.218
2209	35	III	Neg	Neg	2.77	NA
2204	29	III	Pos	>50%	3.27	0.158
2196	30	III	Pos	Neg	NA	0.39
2137	34	III	Neg	>50%	NA	0.04
2105	30	III	Neg	>50%	NA	0.25
2140	38	II	Pos	1-5%	0.76	1.22
2142	50	III	Pos	5-50%	0.08	NA
2197	50	II	Pos	Neg	0.77	NA
2185	51	III	Neg	5-50%	3.20	0.156
2179	51	II	Pos	Neg	0.05	NA
2097	54	III	Pos	>50%	0.82	NA
2021	54	III	Pos	Neg	0.43	NA
2092	59	III	Neg	Neg	0.001	NA
2093	62	III	Pos	>50%	0.19	0.293
2182	64	III	Neg	Neg	0.13	0.265
2022	64	III		1-5%	0.14	NA
2070	49	III	Neg	Neg	NA	0.70
2149	46	III	Pos	Neg	NA	0.83

All values for mRNA expression were taken from RT-qPCR carried out on snap frozen specimens and FFPE tissues. NA: not available.

Chapter 4. Discussion and Conclusions

Although breast cancers in young women (≤35 years) are rare (accounting for about 2.5% of all breast cancers), prognosis is much poorer for young women with the disease (Albain *et al.*, 1994; Kollias *et al.*, 1997). They exhibit differences in their pathological features with breast cancers in older women, which may account for the reported poorer prognosis. These include a higher frequency of grade III cases, a frequent lack of ER and PgR receptors (Ahn *et al.*, 2007; Colleoni *et al.*, 2002), stabilised p53 (Albain *et al.*, 1994; Walker *et al.*, 1996) and higher levels of proliferation (Hartley *et al.*, 2006).

Previous research in our group examined LOH at three chromosomal intervals, containing BRCA1, BRCA2 and TP53. A high incidence of LOH was observed at both BRCA1 and BRCA2 (Johnson *et al.*, 2002). Subsequently, a cDNA microarray study was carried out to gain a global overview of gene expression changes in breast cancers in young women. cDNA from two sporadic tumours from women ≤35 years was compared to normal breast tissue from a reduction mammoplasty and the non-tumourigenic HBL-100 breast cancer cell line. A number of genes that showed increased expression in the breast cancers compared to the controls were identified by SAM analysis as described in the Results (Section 3).

The aim of this project was to investigate the differential expression of 9 candidate genes, first identified in the microarray, in a cohort of breast tumour samples from women of different ages using RT-qPCR, and subsequently for two of these targets, examine protein expression using western blotting and IHC. Although most of the target genes showed some potential, there were some inconsistencies between the gene expression data generated in this project and the primary microarray, and the gene expression profiles found in fixed and snap frozen samples.

4.1 Method development and validation

4.1.1 RT-PCR

The manual RT-PCR study was informative for the nine target genes investigated. In the validation experiments GAPDH showed approximately equal expression across all cell lines enabling the comparison of target gene expression between the cell line samples. Although occasionally the manual RT-PCR and real-time quantitative PCR did not correspond, this most likely arose from the practical limitations of the manual RT-PCR method. For example, there is a relatively narrow dynamic range over which expression can be detected using ethidium bromide staining, which leads to low amplification products being undetectable.

Conversely, reactions risk going beyond the exponential phase of the PCR in achieving detectable amounts of product. It is important that the PCR is ended in the exponential phase if comparisons of relative expression are to be made with any accuracy. This problem is worsened where differences in gene expression are relatively small. For example, the results from DDB2, NCOA3, RARRES3, and RBBP4 all showed slight variations in expression between manual RT-PCR and RT-qPCR. These discrepancies were probably a result of the manual RT-PCR having reached the plateau phase for certain cDNA samples so that differences in expression were no longer easily comparable. Since the RT-qPCR was carried out in triplicate and showed low variation, this is likely to be the most reliable and accurate data.

4.1.2 Housekeeping gene selection

A major issue when studying differential gene expression is how to compare differences between heterogeneous tissue samples. Ideally, mRNA expression is normalised relative to the total number of cells analysed. This approach was not taken in this study as it was not possible to calculate cell numbers from solid epithelial tissues. Another option is to use total RNA quantity as a normaliser (Tricarico *et al.*, 2002), however cellular RNA content can also vary, for example increasing with tumour aneuploidy (Enker *et al.*, 1991). In addition, total RNA quantification does not correct for differences in RNA quality, or reverse transcription efficiency between samples, both of which may have varied between different samples used in this study, particularly for the FFPE tissues.

It is generally accepted that gene expression levels should be normalised to an internal reference gene that shows minimal variation in expression between all samples. In this study, 3 housekeeping genes HPRT1, TFRC, and PGBD were selected following a review of the literature, as they were found to have low variation across samples (de Kok *et al.*, 2005; Janssens *et al.*, 2004; Vandesompele *et al.*, 2002)) and compared to 18S rRNA (a control increasingly used for normalising in RT-qPCR due to its stability (Morse *et al.*, 2005; Pierga *et al.*, 2005; Zhang *et al.*, 2007)), in order to select a suitable reference for subsequent target gene expression analyses. The comparison showed that HPRT1, TFRC, and PGBD displayed similar levels of expression in all samples. By contrast, 18S was expressed at higher levels, and there was markedly more variation in its expression between samples, particularly in the normal organoid samples. Since the expression of HPRT1, TFRC, and PGBD were comparable, and relatively low, they were selected as optimum to control for

RNA quality, and reverse transcription efficiency. For this reason the average of the 3 housekeeping genes was calculated, and used for normalisation in this study. Moreover, this approach of averaging of housekeeping gene expression is increasingly recommended for normalisation in RT-qPCR experiments (Akilesh *et al.*, 2003; Bremer *et al.*, 2007; Meller *et al.*, 2005; Vandesompele *et al.*, 2002).

4.1.3 Differences between microarray and RT-qPCR data

The results of the microarray study showed that the 9 target genes selected for further investigation in this project were up-regulated in the breast tumours in younger women, when compared to normal breast organoids and the HBL-100 breast cell line. These results held true for 6 of the 9 targets by RT-qPCR, however 3 of the nine target genes were shown to be down-regulated in breast tumours from women ≤35 years. There are a number of possible reasons that may explain these differences. One important difference between the microarray and expression experiments is that the target gene expression in the latter was represented as target gene expression normalised against 3 housekeeping genes (relative expression) whereas, in the microarray, target gene expression in the tumour samples was relative to target gene expression in the organoids and the HBL-100 cell line for a fixed amount of starting RNA (5µg), without any internal reference gene normalisation. In addition, since the microarray analysis was only carried out on two tumour samples and the qPCR study examined a total of 15 cases from women ≤35 years, the results from qPCR are arguably more reliable than the results obtained from the microarray, and therefore most likely to provide a more accurate overview of the expression trends of all 9 target genes in breast cancers in young women (≤35 years).

4.1.4 Comparison between FFPE and snap frozen gene expression data

The quantitative analysis of RNA extracted from archival tissues is a relatively new application of RT-PCR technology. While some groups have used this approach with varying success (Cronin *et al.*, 2004; Godfrey *et al.*, 2000; Paik *et al.*, 2004; Specht *et al.*, 2001) there remain several limitations, which may explain why the method is still not widely used. In their study, Paik *et al.* (2004) successfully quantified mRNA expression in 668 of 675 FFPE tissues to detect distant metastasis in breast cancers. Similarly, Specht *et al.* (2001) found comparable results between matched frozen and FFPE tissues following mRNA

quantitation. On the other hand, a different study found that mRNA expression in FFPE tissues was less accurate in comparison to freshly frozen tissues (Godfrey *et al.*, 2000). In this study, the mRNA expression of 9 target genes was measured in 36 breast tumours (23 FFPE and 21 snap frozen). For all 9 target genes, the expression profiles in both age groups showed reasonable correlation between tumours that were persevered by fixation with formalin, versus those that were snap frozen. However, unlike the snap frozen specimens, for the FFPE specimens there was no difference in the expression levels between the two age groups. Of the total 36 breast tumour cases, 9 cases had both FFPE and snap frozen specimens examined, however, the expression profiles of the 9 target genes varied widely in these matched tumour specimens. There are a number of possible explanations for this variation in the expression profiles between matched specimens.

Firstly, the heterogeneous nature of breast cancer results in variation between different parts of the same tumour, therefore a certain degree of variation between different frozen and fixed tissue taken from different areas of the same tumour could be expected. It is known that RNA extraction from FFPE tissues is difficult, as RNA may be degraded prior to, during, and after the fixation process (Mizuno *et al.*, 1998). Fixation causes cross-linking of proteins which hinders RNA isolation from tissues, and mono-methylol addition on all four nuclei acid bases, which can compromise the reverse transcription process (Masuda *et al.*, 1999). There were several factors that determined the RNA quality of the tissues used in this study. These included: the length of fixation and degradation of the RNA during fixing, archive storage time, the freeze-thaw cycles of frozen tissues, and efficiency of RNA recovery. Since this work was done retrospectively, it was impossible to control for archive storage time or duration of fixation (which ranged from 18-36 hours), both of which are known to affect RNA integrity (Cronin *et al.*, 2004). Moreover, some of the frozen cases were used in the past by other members of the group, so a small amount of RNA degradation following repeated freeze-thawing in these cases was also to be expected.

Taking into consideration that only ~3% or less of RNA isolated from paraffin samples is accessible to cDNA synthesis when compared to snap frozen samples (Lewis *et al.*, 2001), that RNA from FFPE tissues is frequently degraded, and that small amplicon sizes have been recommended in RT-qPCR using formalin fixed tissues (Antonov *et al.*, 2005), specific steps were taken in this study to maximise RNA yield, facilitate cDNA synthesis and subsequent

target amplifications in the qPCR reactions. These included the optimisation of PK digestion of tissue, and DNase treatment of RNA. PK digestion works to release RNA from the meshwork of crosslinked molecules, potentially down to the level of tetrapeptides (Kraus *et al.*, 1976). Nevertheless, it does not attack the actual methylene bridge that forms the crosslink (Kraus *et al.*, 1976). While the main aim of using PK was to digest the tissue and release the RNA, the heat applied during the PK digestion incubation can be harmful to RNA. To avoid distortion of target gene amplifications due to residual genomic DNA, all RNA samples were treated with DNAse following isolation.

The efficiency of the cDNA synthesis reaction is crucial when measuring mRNA expression, however cDNA synthesis efficiency from total RNA is rarely at 100% (Newton *et al.* 1997). As it was not possible to carry out all cDNA synthesis reactions simultaneously, there may have been some variation with the efficiencies of the reactions between different batches of samples, particularly in the case the FFPE specimens. Additionally, taking into consideration the fragmented nature of RNA isolated from FFPE tissue, and to maximise qPCR efficiency, all target amplicons in this study were purposely designed to be less than 110 bases pairs in length. Furthermore, previous reports have found that the recovery and reverse transcription of various mRNA targets vary from fragment to fragment, possibly as a result of regulatory proteins bound to, or crosslinked to different parts of the mRNA (Bernstein *et al.*, 1992; Godfrey *et al.*, 2000). In this study, for one case the Ct value of the FFPE specimen was ~4 cycles higher than in the matched snap frozen specimen. This indicates that there was 16 times more RNA accessible to cDNA synthesis in the snap frozen specimen (Godfrey *et al.*, 2000). Presumably the remaining RNA was chemically altered by formalin-fixation and paraffin-embedding and could not be reverse transcribed.

To further boost the efficiency of the cDNA synthesis and maintain consistency across all samples, random hexamers were chosen as the primer for reverse transcription. These were chosen because they allow cDNA synthesis along the entire length of the transcripts, regardless of RNA fragmentation and avoid possible secondary structures such as loops or stems. This should result in a more even representation of the whole RNA sequence (Resuehr & Spiess, 2003). Oligo (dT) primers are more specific and more sensitive than random priming, however there is a 3' bias, and as many RNA samples utilised in this study were fragmented oligo (dT) primers were not suitable. Similarly gene-specific priming is

known to be highly specific; however, this was not possible since the use of separate priming reactions would exhaust the limiting RNA. Recently, a study has shown that use of 15-nucleotide-long primers (pentadecamers) can yield twice as much cDNA as random hexamers (Stangegaard *et al.*, 2006). The increased efficiency resulted in reverse transcription of >80% of the template RNA, whereas random hexamers had a yield of ~40%. The same group found that use of pentadecamers in comparison to random hexamers resulted in 55-72% more genes being detected in a microarray experiments. Hence, for future experiments pentadecamers would be the most appropriate primer for cDNA synthesis reactions.

Despite the various measures taken, discrepancies were observed between qPCR data from FFPE and snap frozen specimens. It is reasonable to suggest that the variations in target gene expression between fixed and fresh tumour samples is a direct result of 1) the poor quality of RNA found in FFPE tissues, which is vital to successful RT-qPCR analysis, and 2) sample heterogeneity between snap frozen and FFPE tissue areas. Therefore, the results from RT-qPCR carried out on snap frozen tissues are more reliable than those from FFPE tissues.

4.2 Results comparisons – Realisation of aims

4.2.1 Cell lines, normal organoids, and frozen tumour tissues

4.2.1.1 AKAP1

The primary microarray analysis indicated that AKAP1 might be up-regulated in younger breast cancers compared to normal breast tissue. In this study, both manual RT-PCR and RT-qPCR showed that expression of AKAP1 was predominantly increased in the breast cancer cell lines when compared to HBL-100. When tumour cases and organoids samples were examined, no significant differences were found, either between age groups, or between tumour and normal tissues for AKAP1. In fact, considerable variation in expression across all specimens was observed. Although breast cell lines act as a model for breast biology, they do not give an accurate impression of the behaviour of normal or cancerous breast tissues. Therefore, the gene expression analysis carried out on tissues provides a more reliable indication of AKAP1 expression in breast cancers. Since there was no difference in the expression of AKAP1 between age groups, it appears it does not have a relationship with

breast cancers in young women, and is more likely to have a function in breast tumours from women of all ages.

With a known role in RNA processing (Trendelenburg *et al.*, 1996), maintaining nuclear integrity signal transduction via the anchoring of PKAs and other enzymes (Steen *et al.*, 2000), it is possible that expression of AKAP1 in normal or tumour cells helps maintain nuclear integrity and cell survival.

4.2.1.2 APRIL

To date, only myeloid leukaemic and Burkitt lymphoma cell lines have been found to display overexpression of APRIL (Mencinger *et al.*, 1998). The microarray analysis showed over-expression of APRIL in breast cancers from young women. RT-qPCR found APRIL over-expression in two tumourigenic breast cell lines (MDA-MB-231, and MDA-MB-468) compared to the non-tumourigenic HBL-100. Conversely, a high expression was also observed in most organoids samples, as well as in most snap frozen tumours with no apparent differences in the expression levels between age groups. This implies that APRIL does not have a specific role in breast cancers in young women, but may have a role in breast tumours in women of all ages. So far, no other studies have examined APRIL expression in the breast. Since APRIL has been implicated in cell cycle, and RNA biosynthesis (Sun *et al.*, 2001), it is possible that the increased expression found in breast tumours and organoid specimens is directly linked to cell maintenance.

4.2.1.3 C/EBP alpha

Despite overexpression of C/EBPα in younger breast cancers being found in the microarray, the results of this study show that C/EBPα was down regulated in breast cancer cell lines and all breast tumours regardless of age, compared to normal breast tissue. These results are in accordance with another report examining the expression of C/EBPα in breast cancer. Gery *et al.* (2005) found a down regulation of C/EBPα mRNA and protein in primary breast tumours and showed that restoration of C/EBPα expression in a breast cell line resulted in inhibition of growth associated with a G0-G1 cell cycle arrest. Additional studies have also implicated C/EBPα as a tumour suppressor in acute leukaemia (Pabst *et al.*, 2001), head and neck cancer (Bennett *et al.*, 2007), and lung cancer (Halmos *et al.*, 2004). Several studies

investigating the anti-proliferative effects of C/EBPα, have confirmed its interaction with several cell cycle components such as p21 (Timchenko *et al.*, 1996), Rb (Chen *et al.*, 1996), cdk2/4 (Wang *et al.*, 2001), E2F (Slomiany *et al.*, 2000), and SWI/SNF (Muller *et al.*, 2004). There is no published data to explain how C/EBPα expression may be suppressed in breast cancers. However, while studying the tumour suppressor effects of C/EBPα in head and neck tumours, Bennett *et al.* found that C/EBPα is down-regulated by gene promoter methylation (Bennett *et al.*, 2007). It is therefore possible that C/EBPα is also down regulated in breast cancers by gene promoter methylation.

4.2.1.4 DDB2

DDB2 is involved in global genomic repair and nucleotide excision repair, especially involving repair of UV-induced DNA damage (Hwang *et al.*, 1999). DDB2 is expressed in a TP53-dependent manner (Hwang *et al.*, 1999), but can also be expressed independently of TP53 via wild-type BRCA1 (Hartman & Ford, 2002). The primary microarray analysis showed over-expression of DDB2 in breast cancer from young women. In this study, RT-qPCR analysis found DDB2 to be over-expressed in the three ER-positive breast cancer cell lines (MCF-7, ZR-75-1, and T47-D) with much lower expression in the remaining tumourigenic cell lines (MDA-MB-231, and MDA-MB-468). Both MDA-MB-231, and MDA-MB-468 are known to harbour TP53 mutations, hence these results are consistent with the study that has shown DDB2 expression is TP53-dependent (Hwang *et al.*, 1999). MCF-7, T47-D and ZR-75-1 showed increased expression of DDB2. However, DDB2 was increased in T47-D which also has a TP53 mutation; the common link between these 3 cell lines is that they are ER-positive, suggesting other mechanisms are involved.

In the tumour cases, DDB2 was generally expressed at low levels in comparison to the organoid samples. However there was some variability as some tumour from women >35 years showed high DDB2 expression. Loss of DDB2 has obvious tumourigenic potential, with impaired global genomic repair leading to the accumulation of other mutations, promoting the progression to cancer. A recent study found that c-myc has the ability to down regulate p53 target genes such as DDB2 (Ceballos *et al.*, 2005). Since, c-myc is amplified and overexpressed in 15-25% of breast tumours (Deming *et al.*, 2000), it is therefore possible that the down regulation of DDB2 in breast tumour samples observed in this study is a result of deregulated c-myc expression. In the future it might be interesting to examine the

expression of c-myc in the tumour tissues used in this study to determine whether there is a correlation between DDB2 and c-myc.

4.2.1.5 Granulin

The results of this study show that granulin was expressed at high levels in all 5 tumourigenic breast cell lines, particularly in MDA-MB-231, MCF-7, and T47-D. Compared to the organoids samples, granulin was expressed at significantly higher levels in breast tumours from women ≤ 35 years old. There was some variation in the expression levels in tumours from women >35 years old, although the relative expression levels were predominantly high. The results from the RT-qPCR experiments therefore suggest an important role for granulin in breast cancer. Since granulin and its precursors function as autocrine growth factors promoting proliferation (He & Bateman, 2003), as well as cellular processes crucial to cancer progression such as invasion, and metastasis (Cheung *et al.*, 2004), it is not surprising to see high levels of granulin expression in these breast tumour specimens. These results support the findings of (Lu & Serrero, 2000) who found high levels of granulin mRNA expression in MCF-7, and T47-D. The same group also found that granulin is important for proliferation, tumourigenicity, and that oestrogen stimulates granulin expression which in turn stimulates cyclin D (Lu & Serrero, 2001). A pathology study has identified a correlation between granulin protein expression and Ki-67 proliferation index, p53 positive cells, and high grade breast cancers (Serrero & Ioffe, 2003). No correlation between granulin expression and grade was found in my study. In the future it would be worth validating this target using a larger series of cases, given the evidence that exists that indicates a correlation between granulin and high proliferation index, p53 positive cells, and high grade breast tumours (Serrero & Ioffe, 2003), all of which are apparent in breast cancers in younger women. Further work in the area of breast cancer has confirmed that granulin stimulates the expression of MMP9 and VEGF, thereby promoting angiogenesis and metastasis (Tangkeangsirisin & Serrero, 2004).

4.2.1.6 NCOA3

Interest in NCOA3 and its role in breast cancer has grown since it was first shown to be overexpressed in ~60% of breast cancers (Anzick *et al.*, 1997). Several studies have reported

its expression in breast as well as other cancers (Ghadimi *et al.*, 1999; Henke *et al.*, 2004; Rangel *et al.*, 2006), where increased expression is a result of transcriptional up-regulation or amplification (Anzick *et al.*, 2003; List *et al.*, 2001; Reiter *et al.*, 2001). Since NCOA3 is known to interact with steroid receptors as a coactivator, as well as with several transcription factors, thereby promoting cell proliferation (Louie *et al.*, 2006; Oh *et al.*, 2004; Yan *et al.*, 2006), it is not surprising that NCOA3 has now been classed as an oncogene. One study has associated NCOA3 expression with ER presence (Iwase *et al.*, 2003), while others have found it to be associated with breast tumours negative for steroid receptors but positive for HER-2 (Bouras *et al.*, 2001; Kirkegaard *et al.*, 2007; Osborne *et al.*, 2003). Iwase *et al.* saw better responses to hormonal therapy in patients with NCOA3 positive tumours. On the other hand, others have found NCOA3 to be associated with Tamoxifen resistance (Dihge *et al.*, 2007; Osborne *et al.*, 2003). Given the variability in the reports regarding the different association with NCOA3, it is likely to have pleiotropic effects in breast cancers.

In this study, NCOA3 mRNA expression was high in the ER-positive breast cancer cell lines compared to the ER-negative MDA-MB-231, and MDA-MB-468 and the non-tumourigenic HBL-100. There was increased expression in the MCF-7 breast cancer cell line, in agreement with previous studies that found an overexpression of NCOA3 in MCF-7 (Labhart *et al.*, 2005; Tikkanen *et al.*, 2000). Other studies have found overexpression in MCF-7 as well as ZR-75-1 and T47-D (ER-positive cell lines), again lending support to my findings (Anzick *et al.*, 1997; Magklara *et al.*, 2002). In their study Anzick *et al.* (1997) also examined the frequency and levels of NCOA-3 amplification and overexpression. Among 105 unselected breast tumours, they found NCOA-3 amplification in 9.5% and overexpression in 64% of these tumours. Subsequently, Bautista *et al.* tested a population of 1157 breast and 122 ovarian tumours and observed amplification of NCOA-3 in approximately 4.8% of breast tumours and 7.4% of ovarian tumours (Bautista *et al.*, 1998). Other studies also confirmed that NCOA-3 mRNA was expressed at considerably higher levels in tumour tissues than in normal breast tissues (Bouras *et al.*, 2001; List *et al.*, 2001; Murphy *et al.*, 2000).

One main finding of this study was that NCOA3 mRNA levels were significantly higher in breast cancers in women ≤ 35 years old compared to those from women >35 years. The levels were also significantly higher in younger women compared to the normal breast organoids.

Subsequent protein analysis using western blotting supported the RT-qPCR, with the trends observed for protein expression in the breast cell lines following that of the mRNA expression.

However, IHC examination of 56 breast tumours found significant variation in staining with no significant difference in NCOA3 protein expression between tumours from younger and older women. Only 6 out of 20 tumours from women ≤35 years showed NCOA3 expression, compared to 8 out of 15 in tumours from women aged 36-49, and 12 out of 21 in the older age group (≥50 years). Moreover, there was no correlation between the mRNA and protein expression in these cases. There was also no relationship with ER status.

Given the evidence that NCOA3 acts as an ER coactivator, it is perhaps not surprising to find overexpression in the ER-positive cell lines and tumours. However, it is also not surprising to see expression in ER-negative tumours since 3 studies have found NCOA3 to be associated with breast cancers lacking steroid receptors (Bouras *et al.*, 2001; Kirkegaard *et al.*, 2007; Osborne *et al.*, 2003). Since NCOA-3 may act as a coactivator for nuclear receptors such as ER, and NCOA-3 amplification correlates with a positive ER status in breast cancers, NCOA-3 may be an important contributor to estrogen-mediated tumour growth. However, accumulating evidence now indicates additional roles for NCOA-3 in breast tumourigenesis independent of ER, since overexpression has not always correlated with ER- and PR-positive tumours. For example, Bouras *et al.* observed NCOA-3 overexpression in 26 of 85 (31%) invasive breast tumours, which included overexpression in 15 of 26 (58%) ER-negative tumours and in 11 of 55 (20%) ER-positive tumours (Bouras *et al.* 2001). Likewise for PR, NCOA-3 overexpression occurred more frequently in PR-negative tumours than in PR-positive tumours used in this study. Furthermore, the same study found that NCOA-3 overexpression in these invasive breast tumours was correlated with high levels of HER2/neu and p53 proteins (the latter a common feature of breast cancers in young women). Another study confirmed not only a role for NCOA-3 in ER-dependent and ER-independent breast cancer proliferation, but also that NCOA-3 overexpression may leave ER positive cells resistant to anti-oestrogens (Louie *et al.*, 2004). The same study also found a role for NCOA-3 in cell cycle control.

The fact that NCOA3 was found to be expressed at the mRNA level but not at the protein level in some tumours in this study is perhaps unexpected. This may be due to an insertion of a stop codon in the sequence, or the presence of specific micro-RNAs that may block protein

translation (O'Donnell *et al.*, 2005; Petersen *et al.*, 2006; Zeng *et al.*, 2003). Recently the Mir-17-5p micro-RNA was found to down-regulate NCOA3 primarily through translational inhibition (Hossain *et al.*, 2006). Therefore, it would be interesting to check for a role for Mir-17-5p in the breast tumours that showed reduced or absent NCOA3 protein and compare young and older cases.

The inconsistencies between mRNA and protein expression in the same cases could also be a result of differences in sensitivity of the assays as there was only a weak reactivity of the NCOA3 antibody used despite careful method optimisation.

4.2.1.7 RARRES3

RARRES3 may promote cell differentiation and growth suppression (DiSepio *et al.*, 1998). Not surprisingly, decreased expression is seen in many cancers including colorectal adenocarcinoma (Shyu *et al.* 2003), basal cell carcinoma (Duvic *et al.*, 2003), B-cell lymphocytic leukaemia (Casanova *et al.*, 2001), hepatocellular carcinoma and cholangiocarcinoma (Jiang *et al.*, 2005a). In contrast, RARRES3 mRNA has recently been found to be overexpressed in breast cancer (Shyu *et al.*, 2005). In their study, Shyu *et al.* found a significant correlation between low RARRES3 mRNA expression and ER-positive breast cancers. The study also showed that RARRES3 is down-regulated by oestrogen in ER-positive breast cell lines.

In my study, analysis of breast cancer cell lines found high RARRES3 expression in the ER-negative MDA-MD-468, low levels in MDA-MB-231, and RARRES3 was absent in all other cell lines, in keeping with the report by Shyu *et al.* (2005). RARRES3 expression was also found to be significantly higher in breast tumours from ≤ 35 years compared to older women and organoid samples. Unfortunately, there were too few cases available to enable correlation with ER status. However, there was an equal spread of ER-positive and ER-negative cases with high RARRES3 expression. Given its growth suppressive activity (DiSepio *et al.*, 1998; Tsai *et al.*, 2006), it is perhaps surprising to see an over-expression in breast cancer. In addition, although it is expressed at the mRNA level, there are as yet no reports in the literature to suggest a functional role in breast cancer or other cancer types. Unfortunately, there was no commercially available RARRES3 antibody applicable for IHC at the time of investigation. In the future, it would be interesting to examine protein

expression and the interaction with the ER, if any, to gain insight into the role of RARRES3 in breast carcinogenesis, and in particular in tumours from young women (≤35 years).

4.2.1.8 RBBP4

The primary microarray analysis showed over-expression of RBBP4 in breast cancers from young women. RT-qPCR showed expression of RBBP4 in MDA-MB -231, MDA-MB-468, ZR-75-1 and T47-D, but no expression in MCF-7 and HBL-100. RBBP4 was expressed at high levels in the majority of tumour specimens and organoids samples, with no apparent difference in expression levels between the age groups. To date, increased RBBP4 expression has not been found in breast cancers. Increased expression has been found in thyroid (Pacifico *et al.*, 2007), hepatocellular (Song *et al.*, 2004), and non-small cell lung (Fukuoka *et al.*, 2004) cancers, and a down-regulation seen in mucoepidermoid carcinoma (Leivo *et al.*, 2005). Since RBBP4 was first identified as a retinoblastoma binding protein, it is likely that its function in tumourigenesis is cell cycle related. However, no evidence has been put forward to support this. However, in thyroid cancer there is an indication that RBBP4 supports proliferation through nuclear factor-kappa B (Pacifico *et al.*, 2007). It is therefore possible that the increased expression of RBBP4 seen in breast tumours in this study may have a similar tumourigenic effect.

4.2.1.9 TGFβI

The microarray study identified TGFβI as a potential novel target up-regulated in breast cancers in young women. However, the results of the qPCR do not support those findings, as RT-qPCR found a higher expression of TGFβI in the organoids samples compared to all tumours regardless of age. The non-tumourigenic HBL-100 breast cell line also had higher levels of expression compared to the tumourigenic cell lines, although MDA-MD-231 and MCF-7 did show low levels of expression.

TGFβI has been found to be overexpressed in several cancers including squamous cell carcinomas (Hu *et al.*, 2001; Tomioka *et al.*, 2006), oesophageal (Hourihan *et al.*, 2003), pancreatic (Schneider *et al.*, 2002), colorectal (Buckhaults *et al.*, 2001), as well as in ovarian endometriosis (Arimoto *et al.*, 2003). However, reduced expression has been observed in ovarian cancer cell lines (Walker *et al.*, 2007), and lung carcinoma (Zhao *et al.*, 2006).

Functional analysis has shown that TGFβI is secreted into the ECM (LeBaron *et al.*, 1995) where it has the ability to interact with matrix components such as fibronectin, laminin, as well as several collagen types (Kim *et al.*, 2002). One study showed that TGFβI supports adhesion, migration and proliferation of renal tubular epithelial cells (Lee *et al.*, 2003). However, it has also been found to act as an attachment protein (LeBaron *et al.*, 1995). Although there is conflicting evidence regarding TGFβI's function with regard to the tumourigenesis (either as a pro-tumourigenic or anti-tumourigenic agent), it is possible that the down-regulation observed in this study is a result of defective signalling processes in breast cancer that usually work to maintain normal cell architecture.

In view of its ability to interact with integrins, and other ECM molecules, and to support invasion and migration of cells, TGBI could be involved in various aspects of tumourigenesis, including tumour progression, angiogenesis and metastasis. Further investigations of TGFβI and its ability to interact with growth factors such as VEGF and their receptors which are closely associated with breast tumourigenesis is an obvious area for further study.

4.3 The possible relevance of the 9 gene targets to breast cancers in young women

Breast cancers in young women (≤35 years) have a much poorer prognosis compared to tumours from women aged >35 years, and this is believed to be due to differences in the tumour biology. The assessment of 9 target genes in this study, has led to the identification of 2 targets (NCOA3 and RARRES3) that may have a particular importance in breast cancers in young women (≤35 years), since both genes showed higher levels of mRNA expression in tumours from young women compared to those from older women. This is a novel finding which has not been demonstrated previously. Given the pleiotropic functions of NCOA3, it is possible that it plays a role in the evolution of the characteristic pathological features of breast cancers in young women (high grade, p53/ER/PgR/HER-2 status, and high proliferation index). Although RARRES has proposed tumour suppressor functions, it is not yet clear whether it maintains this tumour suppressor function in breast tissue and functional studies are needed to investigate this.

The remaining 7 target genes do not appear to relate specifically to breast cancers in young women, as all 7 targets displayed similar levels of mRNA expression between tumours from

younger and older women. AKAP1 showed similar levels of expression in both normal and breast tumour tissues of all ages. APRIL, RBBP4, and granulin showed increased expression in tumour from women of all ages, whereas three genes (C/EBPα, DDB2, and TGFβI) showed a down-regulation in tumour samples regardless of age.

4.4 Conclusions and future studies

This study has highlighted a number of novel gene targets that show differences in mRNA expression between breast cancer cell lines and tumour tissues stratified by age. These data have confirmed and extended primary microarray data by showing predominantly increased expression of AKAP1, APRIL, Granulin, NCOA3, RARRES3, and RBBP4. NCOA3 and RARRES3 showed differential expression in breast cancers from young women (≤35 years) compared to older cases

For AKAP1, APRIL, Granulin, and RBBP4 the increased expression was present in tumours from women of all ages hence these can not be considered as markers for breast cancer in young women. However, beyond this study it might be interesting to explore whether or not they have any functional significance in breast cancers by examining the expression of their proteins using western blotting and IHC, and in cell lines using gene knock-out with specific antibodies or siRNA technology. In the case of granulin, however, it would be interesting to validate mRNA or protein expression in a larger cohort of breast tumour as there is published evidence that associates granulin with high proliferation index, positive p53, and high grade tumours (Serrero & Ioffe, 2003), all of which are common features of sporadic breast tumours in young women.

In the case of NCOA3, there was differential expression at the mRNA level between tumours from women ≤35 years and those >35 years. Although the difference was significant at the mRNA level, the frequency of NCOA3 protein expression did not differ between the age groups. In addition, there was poor correlation between mRNA and protein expression in individual samples. In the future it might be interesting to examine the reasons for this, including analysis of the potential roles for microRNAs and/or DNA methylation in silencing of the NCOA3 gene. Another approach would be to use a different antibody for IHC to verify the protein expression in the series of 56 breast tissues used for IHC.

The difference in RARRES3 mRNA expression between tumours from women ≤35 and those >35 is very interesting given the proposed function of RARRES3 as a growth suppressor. To verify whether or not RARRES3 has an important functional role in sporadic breast cancers in young women, it would be necessary to first examine the levels of the protein in a series of tumour cases using IHC before any definite conclusion is made regarding its importance in breast cancers.

In contrast to the microarray, C/EBPα, DDB2, and TGFβI were, on the whole, found to be down regulated in breast tumours of all ages. Given their proposed functions as tumour suppressor, DNA repair, and cell adhesion proteins, it is perhaps not surprising to see these genes were found to be expressed at low levels. As for the other novel target genes investigated in this project, to verify a role in breast cancer it would be necessary first to examine protein expression. Analysis of the C/EBPα promoter would also be another interesting avenue to explore since the promoter has been found to be hypermethylated in head and neck tumours (Bennett *et al.*, 2007). Recently, another PhD student in the group has examined the expression of DDB2 and TGFβI proteins in the same series of breast cancers using IHC, with a decreased expression of both proteins found compared to normal breast tissues. In the future, analysis of the interaction of DDB2 with the c-myc oncogene may identify an important association for some breast cancers. For both DDB2 and TGFβI, functional assays using gene knock-in by transfection to look at proliferation, apoptosis, and invasion would give an insight into the potential role of these targets in breast cancers.

In conclusion, this study has extended previous findings by the research group and has generated novel data to support the hypothesis that sporadic breast cancers in younger women may arise due to specific genetic alterations. NCOA3 and RARRES3 have been identified as two new targets of potential relevance to breast cancer in young women, while the 7 other target genes selected appear to be de-regulated in breast cancers as a whole.

Appendices

Appendix I Summary of the clinical information for all tumour cases.

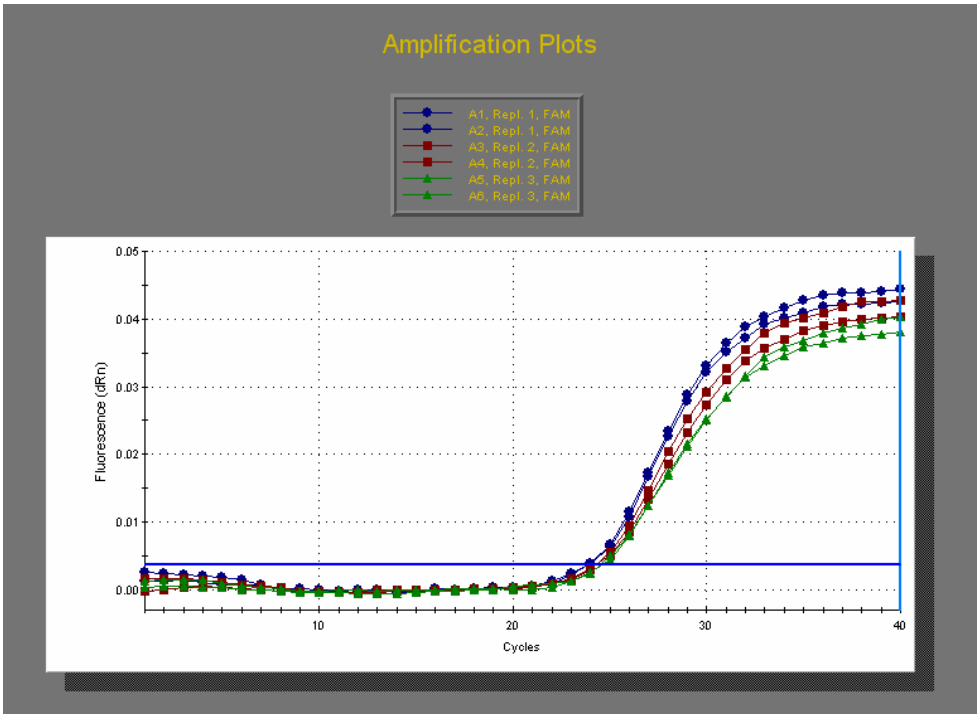
RW No	Age	Size	Type	Grade	Node	ER	PgR
2157	19	1	IDC	III	Neg	Pos	Pos
2195	22	2	IDC	III	Pos	Neg	Neg
2215	25	2	IDC	III	Pos	Pos	Pos
2151	28	2	IDC	III	Neg	Neg	Neg
2144	30	2	IDC	III	Neg	Neg	Neg
2019	31	1	IDC	III	Pos	Pos	Pos
2201	32	2	IDC	III	Neg	Pos	Pos
2204	34	2	IDC	III	Pos	Neg	Pos
2209	35	1	IDC	III	Neg	Neg	Neg
2216	35	2	IDC	II	Pos	Pos	
2204	29	1	IDC	III	Neg	Pos	Pos
2196	30	1	IDC	III	Pos	Pos	Pos
2137	34	1	IDC	III	Neg	Neg	Neg
2134	32	1	IDC	II	Neg	Pos	Pos
2105	30	1	IDC	III	Pos	Neg	Neg
1962	35	1	IDC	II	Neg	Pos	Pos
1932	30	1	IDC	III	Neg	Pos	Pos
1889	31	2	IDC	III	Pos	Pos	Pos
1857	34	2	IDC	II	Pos	Pos	Pos
2190	35	2	IDC	III	Pos	Neg	Neg
2191	34	1	IDC	III	Neg	Pos	Pos
2192	31	1	IDC	II	Neg	Pos	Pos
2193	35	2	IDC	III	Pos	Neg	Neg
2194	33	2	IDC	II	Pos	Pos	Pos
2220	34	1	IDC	II	Pos	Pos	Pos
2140	38	2	IDC	II	Neg	Pos	Pos

2142	50	1	IDC	III	Pos	Pos	Pos
2197	50	2	IDC	II	Neg	Pos	Pos
2185	51	2	IDC	III	Pos	Neg	Neg
2179	51	2	IDC	II	Pos	Pos	Pos
2097	54	2	IDC	III	Pos	Pos	Pos
2021	54	2	IDC	III	Pos	Pos	Pos
2092	59	1	IDC	III	Neg	Neg	Neg
2093	62	2	IDC	III	Neg	Pos	Pos
2182	64	2	IDC	III	Neg	Neg	Neg
2022	64	2	ILC	III	Pos		
2070	49	2	IDC	III	Neg	Neg	Neg
2080	47	2	IDC	II	Neg	Pos	Pos
2082	45	1	IDC	III	Neg	Neg	Neg
2087	49	1	IDC	II	Neg	Pos	Pos
2098	46	1	IDC	III	Neg	Pos	Pos
2112	43	1	IDC	III	Pos	Neg	Neg
2111	46	2	IDC	III	Neg	Neg	Neg
2168	46	2	IDC	III	Pos	Pos	Pos
2194	43	2	IDC	III	Pos	Pos	Pos
2161	47	1	IDC	III	Pos	Pos	Pos
2210	38	2	IDC	III	Pos	Pos	Neg
2208	43	2	IDC	II	Pos	Pos	Pos
2198	45	2	IDC	II	Neg	Pos	Pos
2141	56	1	IDC	II	Neg	Pos	Pos
2209	45	1	IDC	III	Neg	Neg	Neg
2175	41	2	IDC	III	Pos	Pos	Pos
2149	46	2	IDC	III	Pos	Pos	Pos
2116	46	2	IDC	III	Pos	Neg	Neg
2060	54	2	IDC	II	Neg	Pos	Pos
2064	52	2	IDC	II	Neg	Pos	Pos
2125	69	2	IDC	III	pos	Neg	Neg
2113	62	2	IDC	III	Pos	Pos	Pos

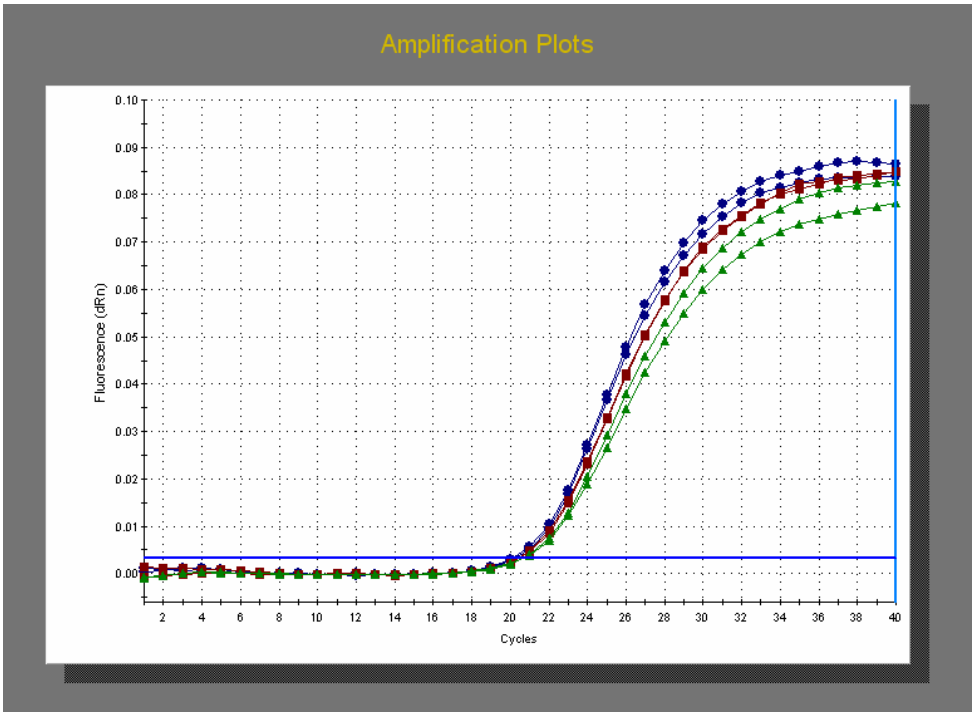
2161	42	2	IDC	III	Pos	Pos	Pos
2159	63	2	IDC	I	Pos	Pos	Pos
2153	54	2	IDC	II	Neg	Pos	Pos
2178	60	2	IDC	II	Pos	Pos	Pos
2180	59	3	IDC	III	Pos	Pos	Pos
2183	64	2	IDC	I	Neg	Pos	Pos
2172	59	2	IDC	III	Neg	Pos	Pos
2170	78	2	IDC	II	Pos	Pos	Pos
2101	40	2	IDC	III	Neg	Pos	Pos
2047	52	2	IDC	III	Pos	Neg	Neg
2139	48	2	IDC	II	Neg	Pos	Pos
2095	51	2	IDC	III	Pos	Neg	Neg
2096	53	2	IDC	III	Neg	Pos	Pos
2074	49	1	IDC	II	Neg	Pos	Pos
2076	54	2	IDC	III	Pos	Pos	Pos

Appendix II Standard curves, dissociation curves, and $MgCl_2$ for RT-qPCR

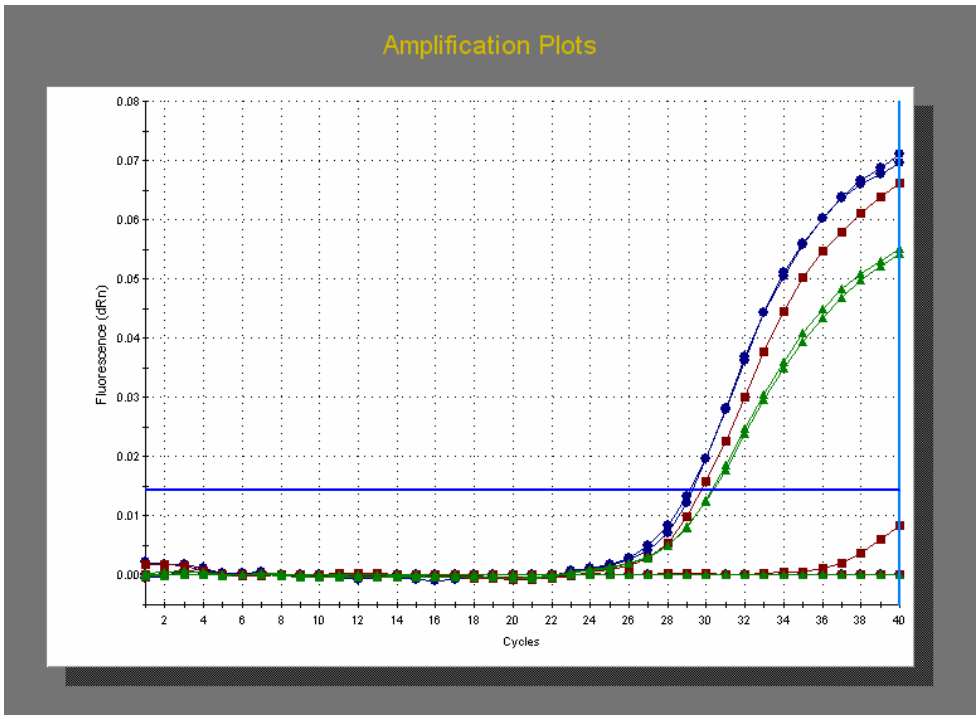
Optimisation of AKAP1 primer pair for $MgCl_2$



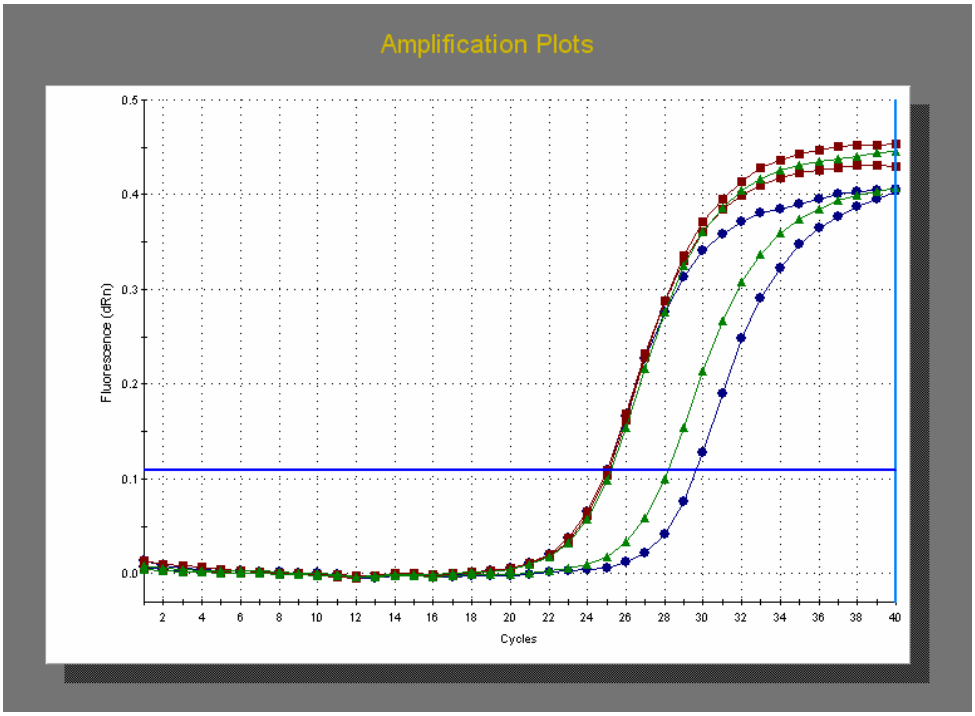
Optimisation of APRIL primer pair for $MgCl_2$



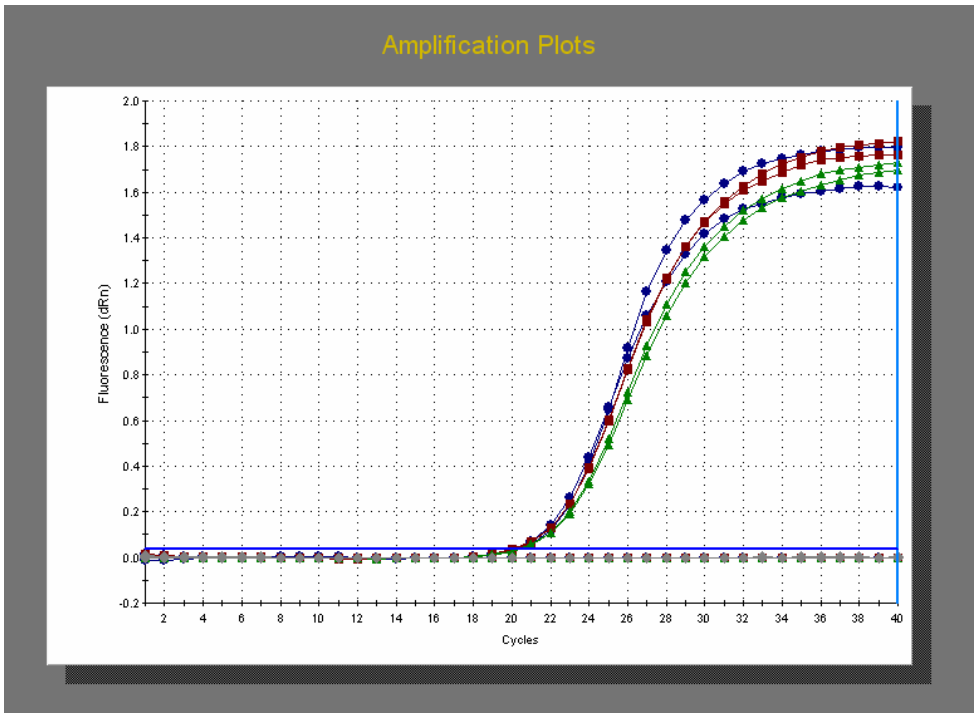
Optimisation of C/EBPalpha primer pairs for MgC l_2



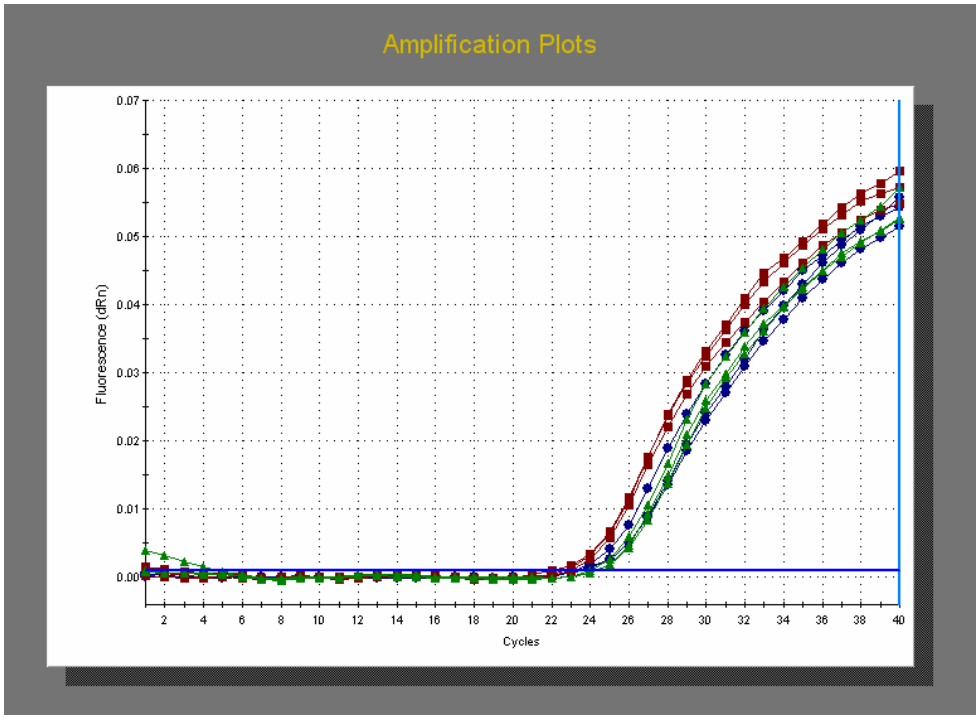
Optimisation of DDB2 primer pairs for $MgCl_2$



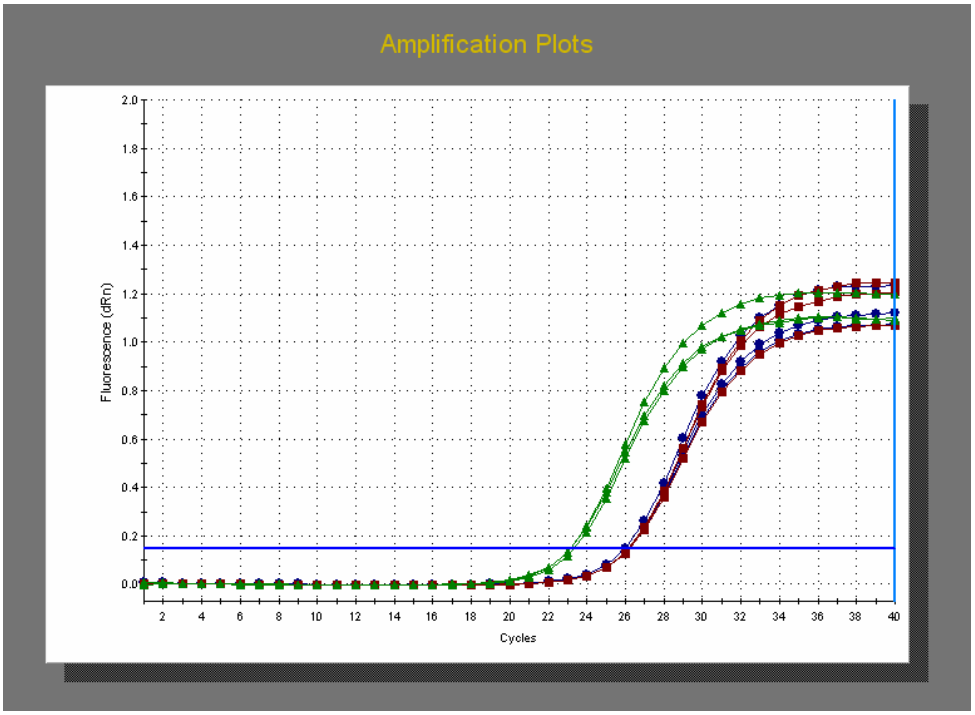
Optimisation of Granulin primer pairs for $MgCl_2$



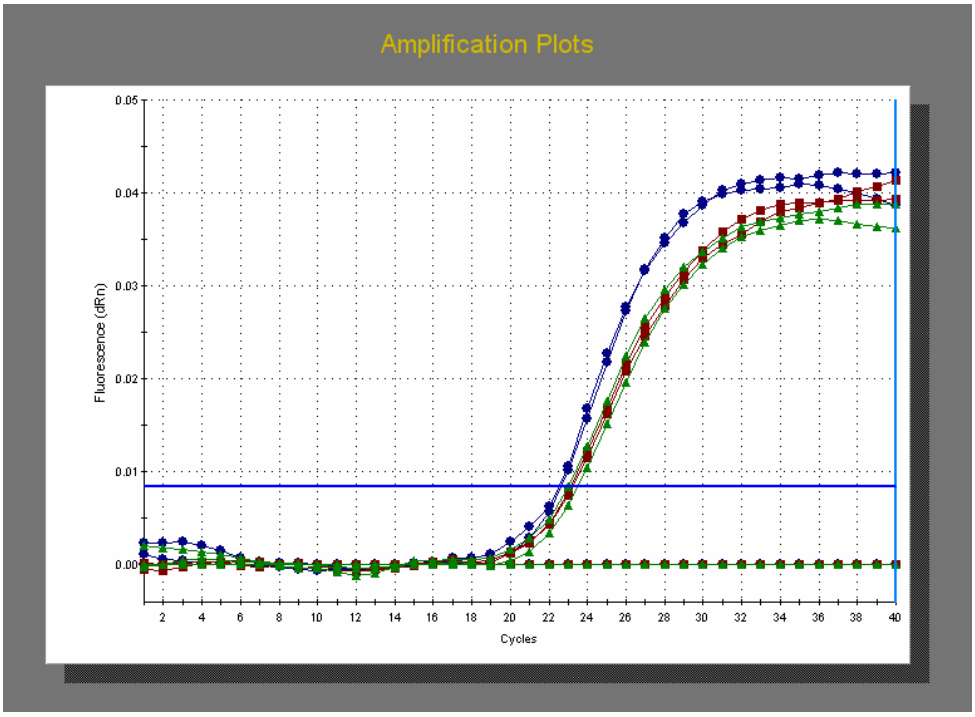
Optimisation of NCOA3 primer pairs for MgC l_2



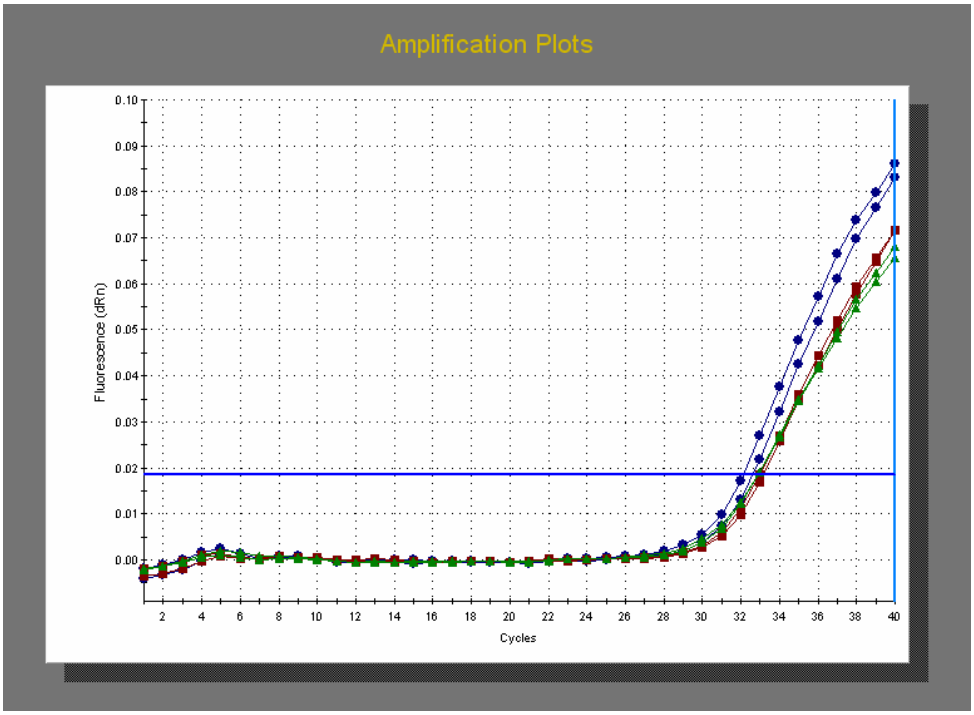
Optimisation of RARRES3 primer pairs for MgCl I_2



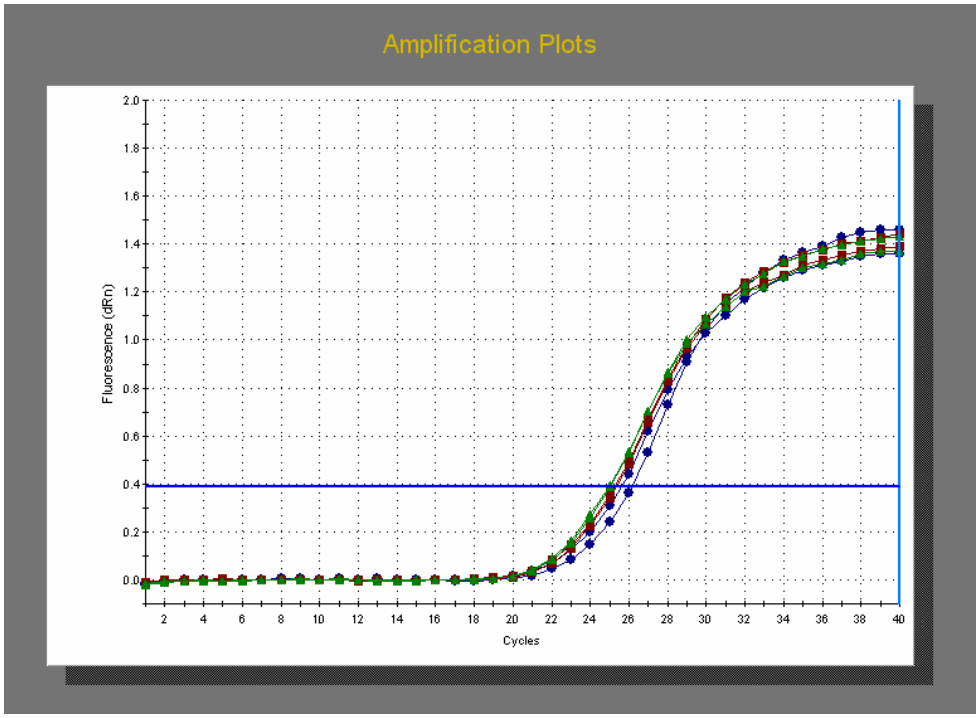
Optimisation of RBBP4 primer pairs for MgCl I_2



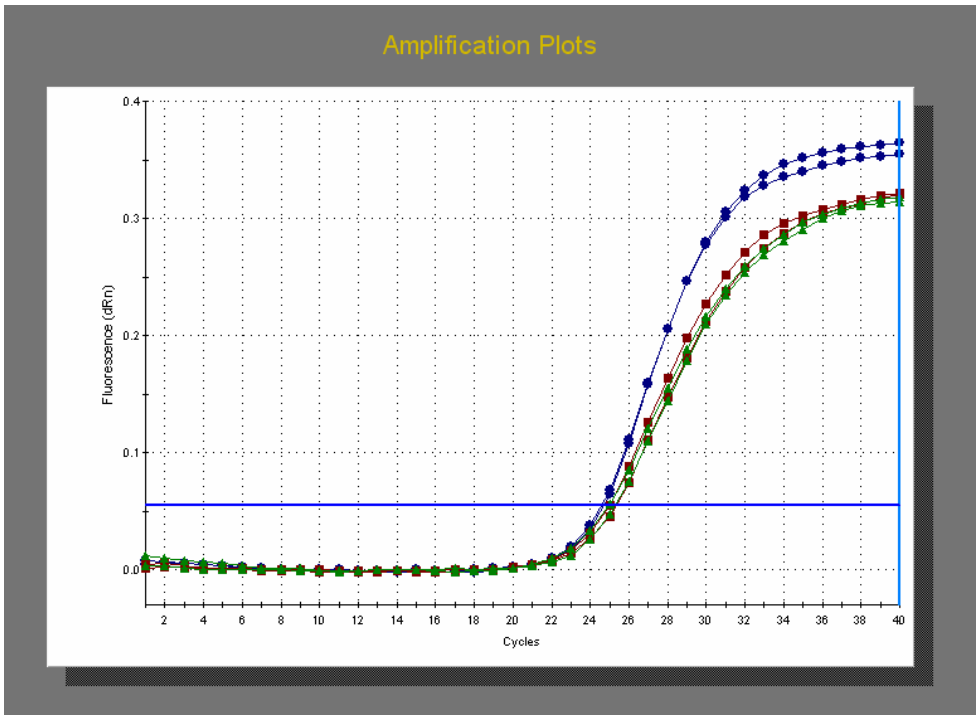
Optimisation of TGFbI primer pairs for $MgCl_2$



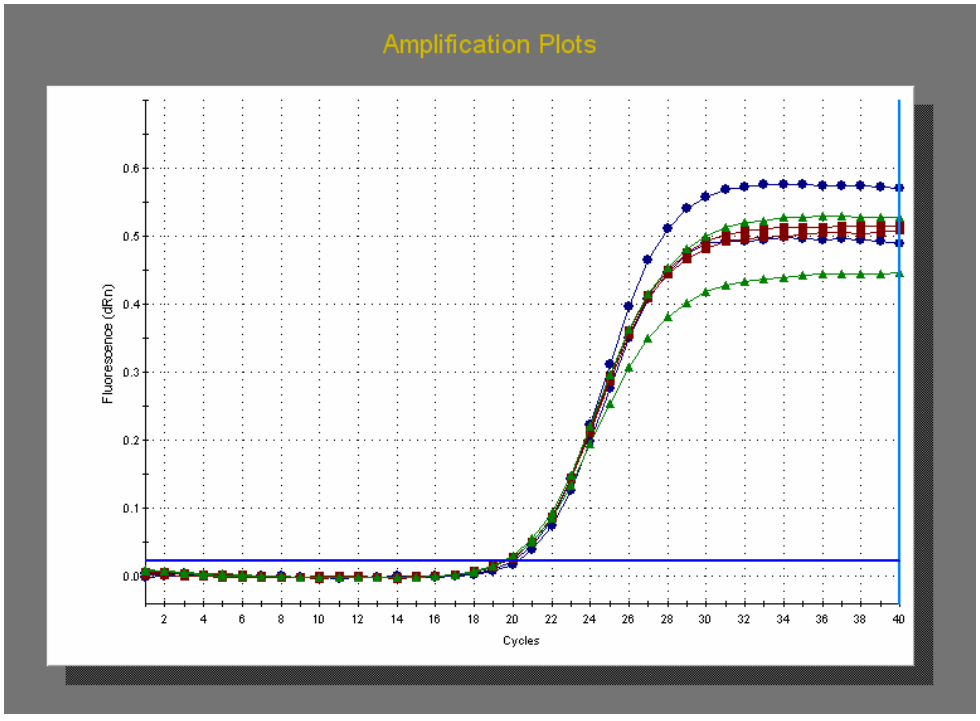
Optimisation of HPRT primer pairs for MgC l_2



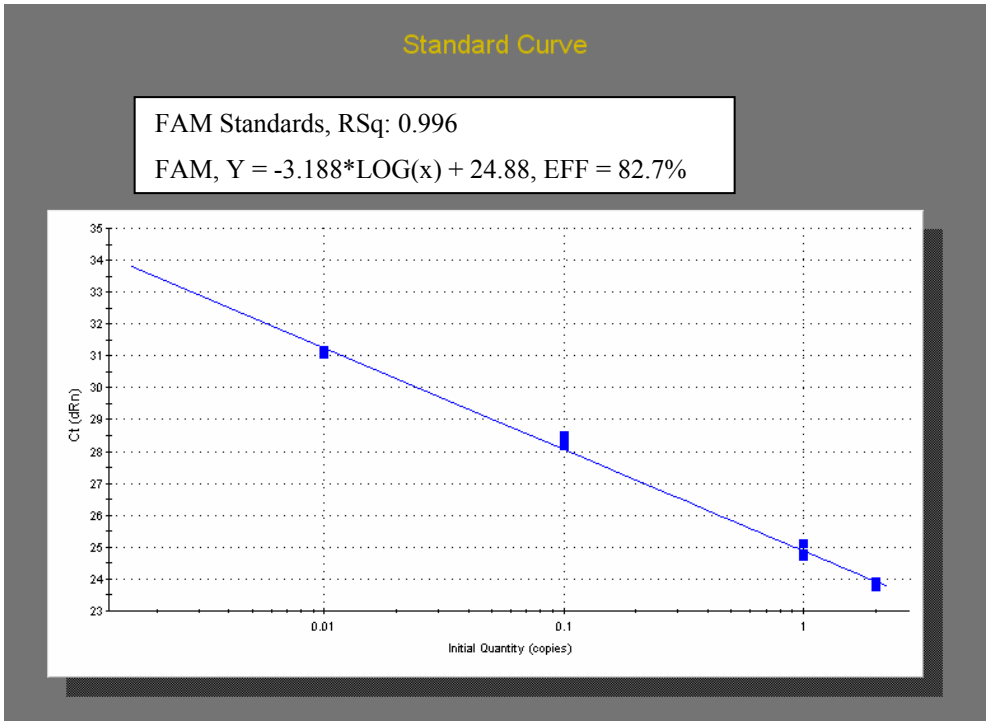
Optimisation of TFRC primer pairs for MgC l_2



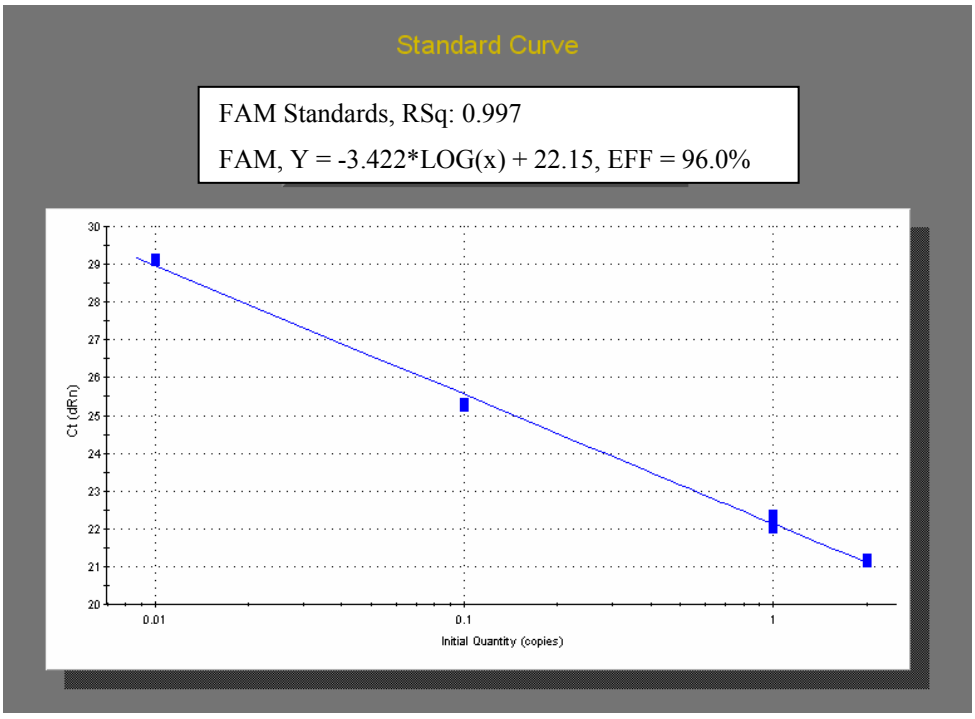
Optimisation of PBGD primer pairs for MgC l_2



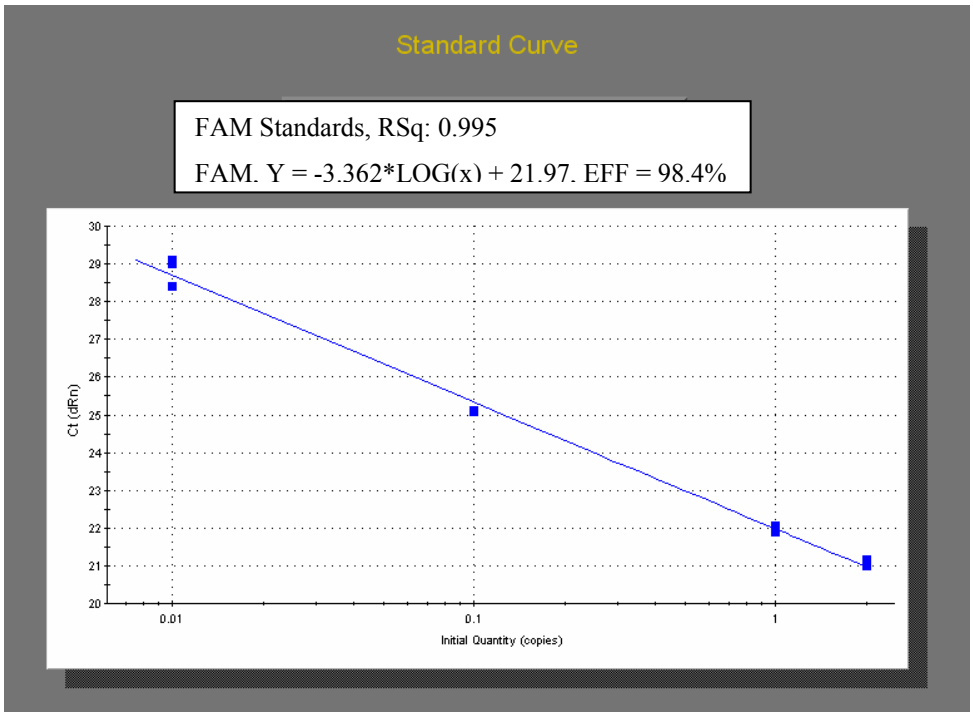
Standard curve for AKAP1



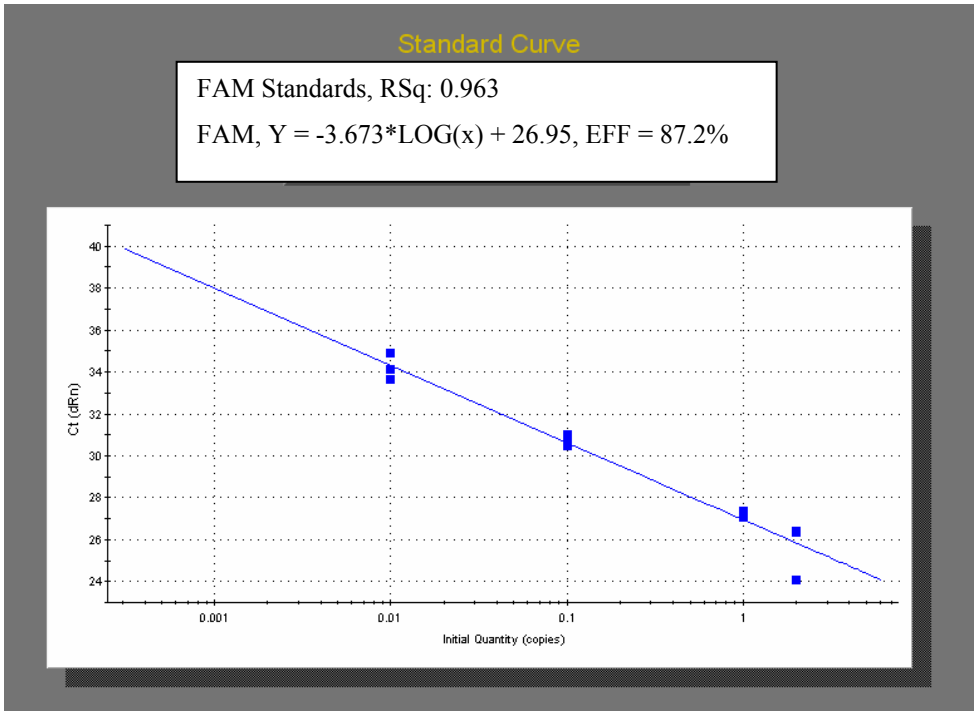
Standard curve for APRIL



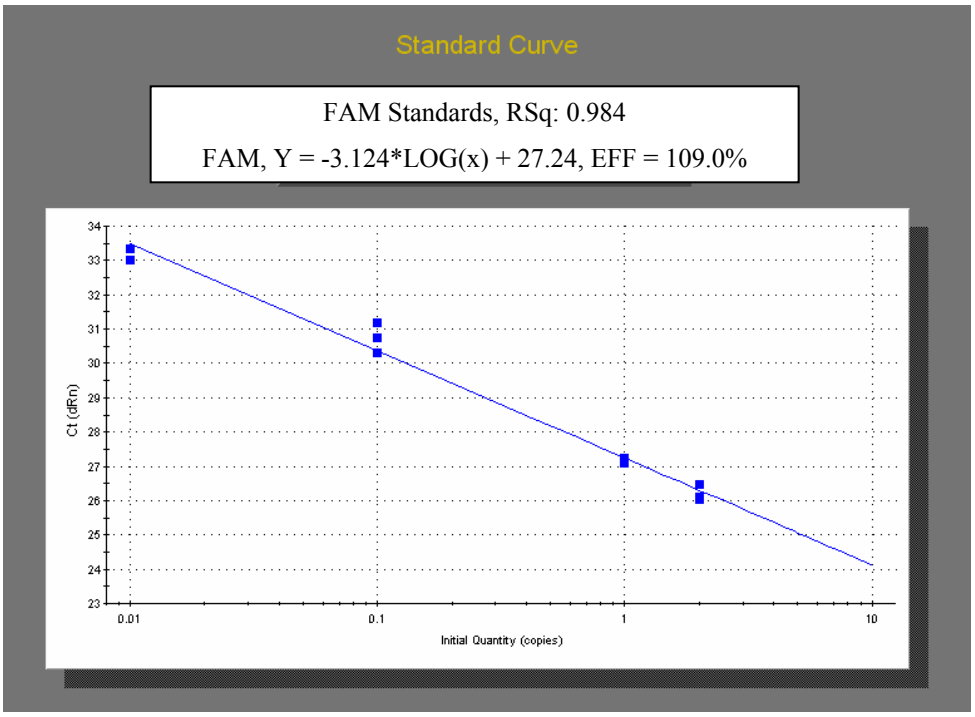
Standard curve for C/EBP alpha



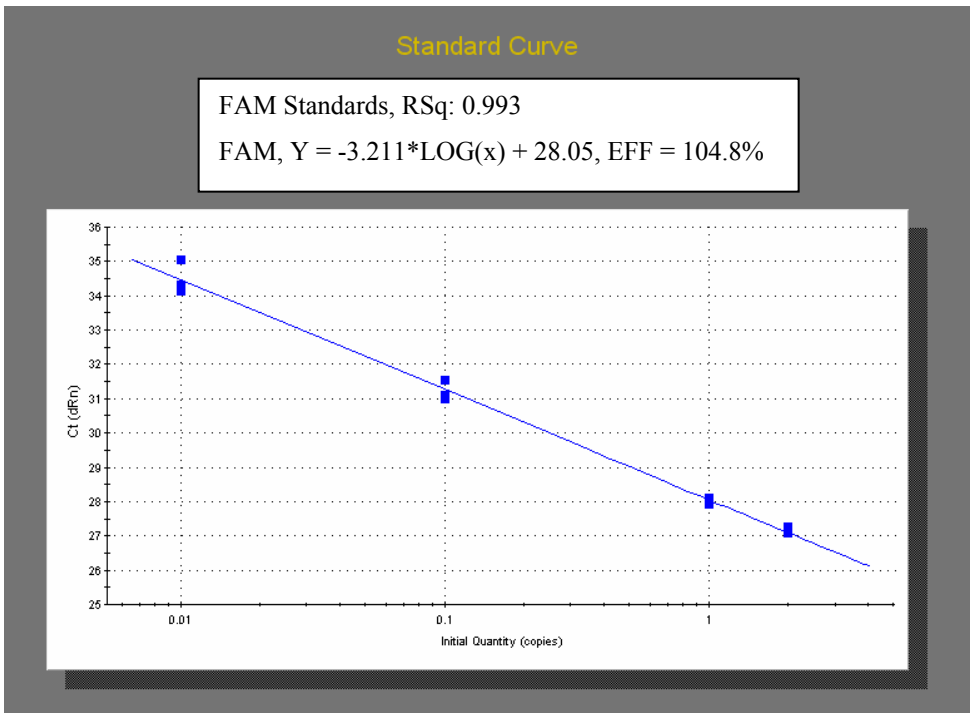
Standard curve for DDB2



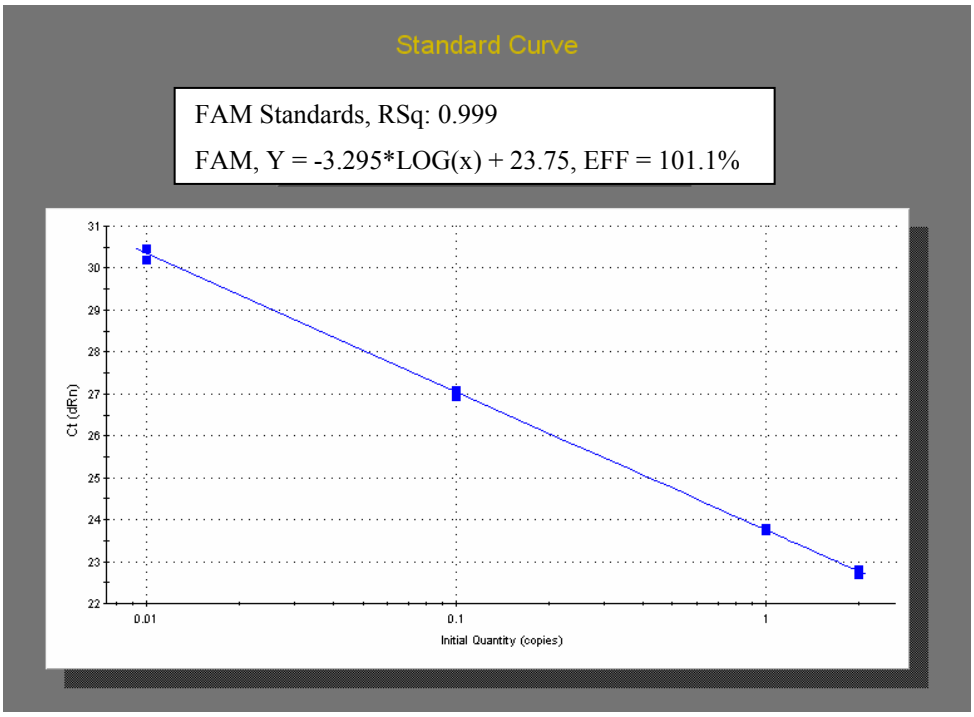
Standard curve for Granulin



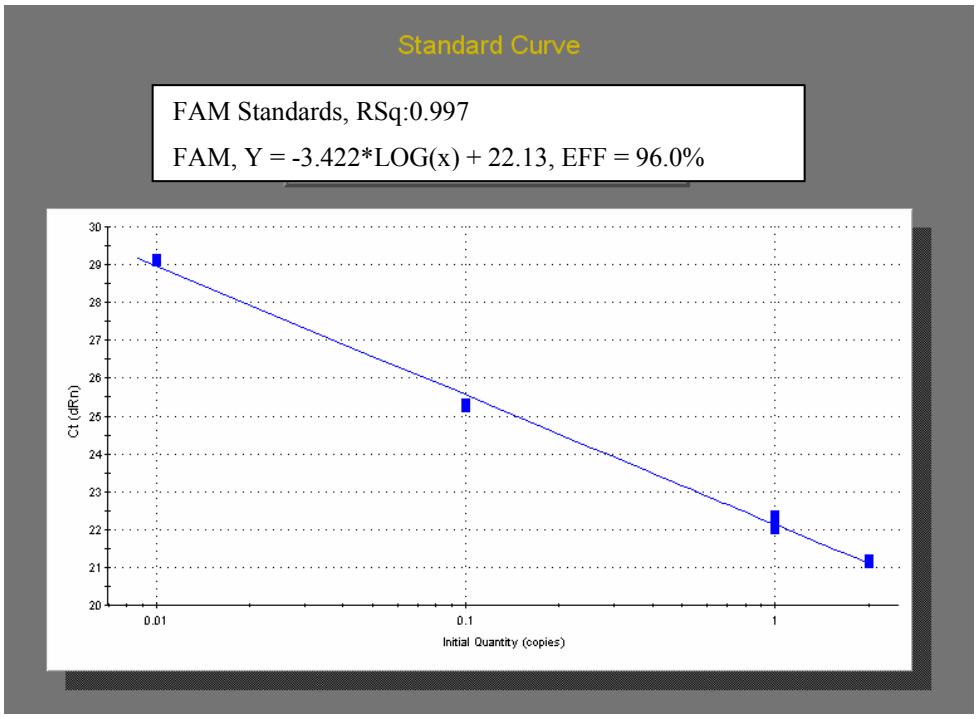
Standard curve for NCOA3



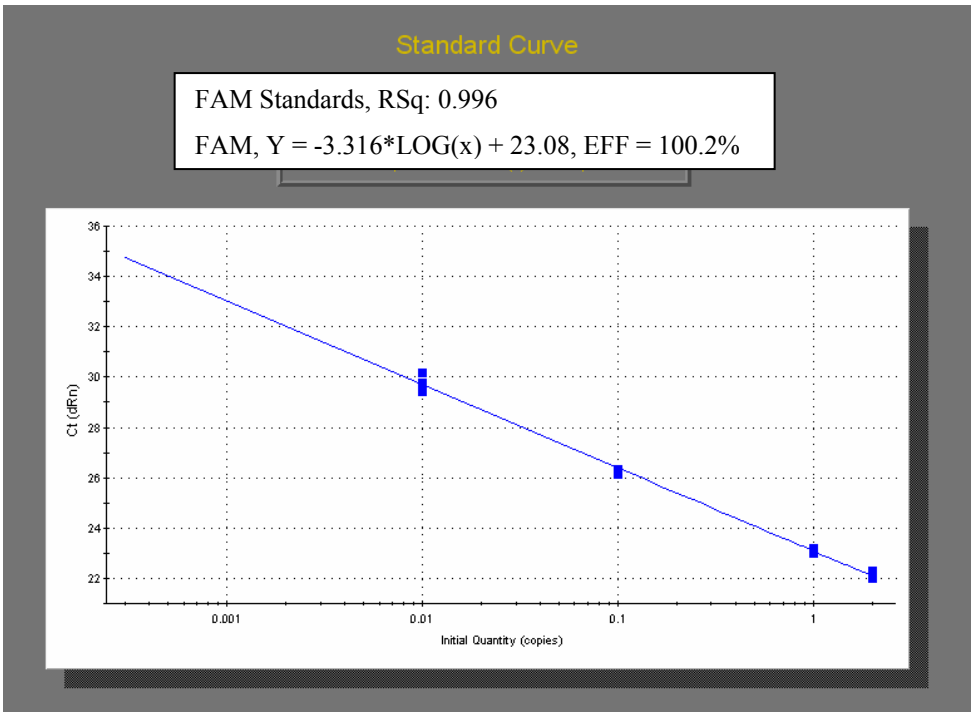
Standard curve for RARRES3



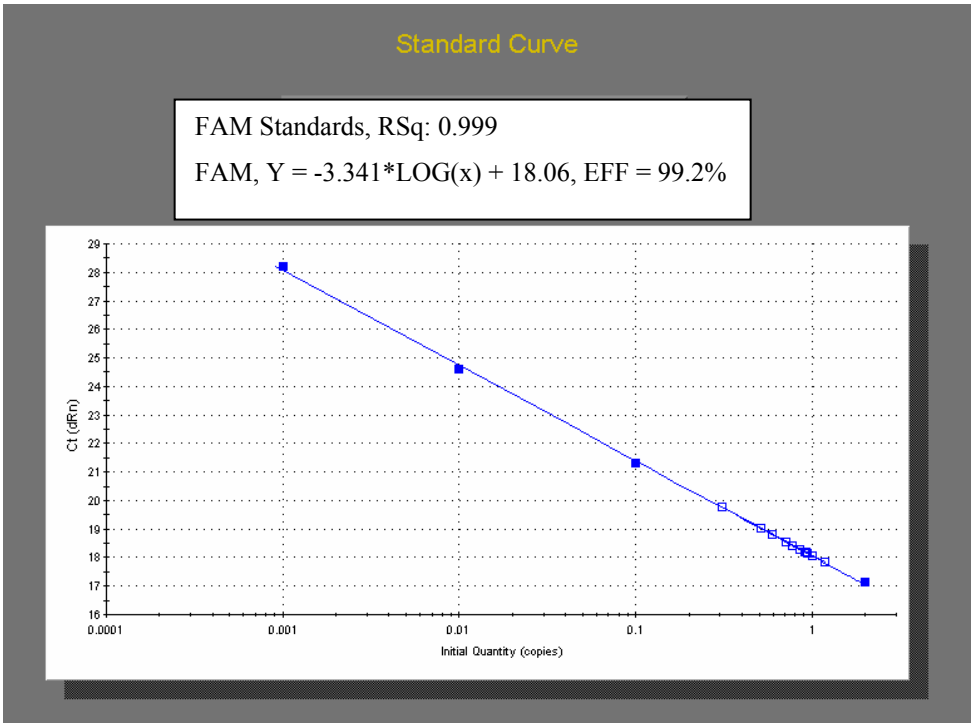
Standard curve for RBBP4



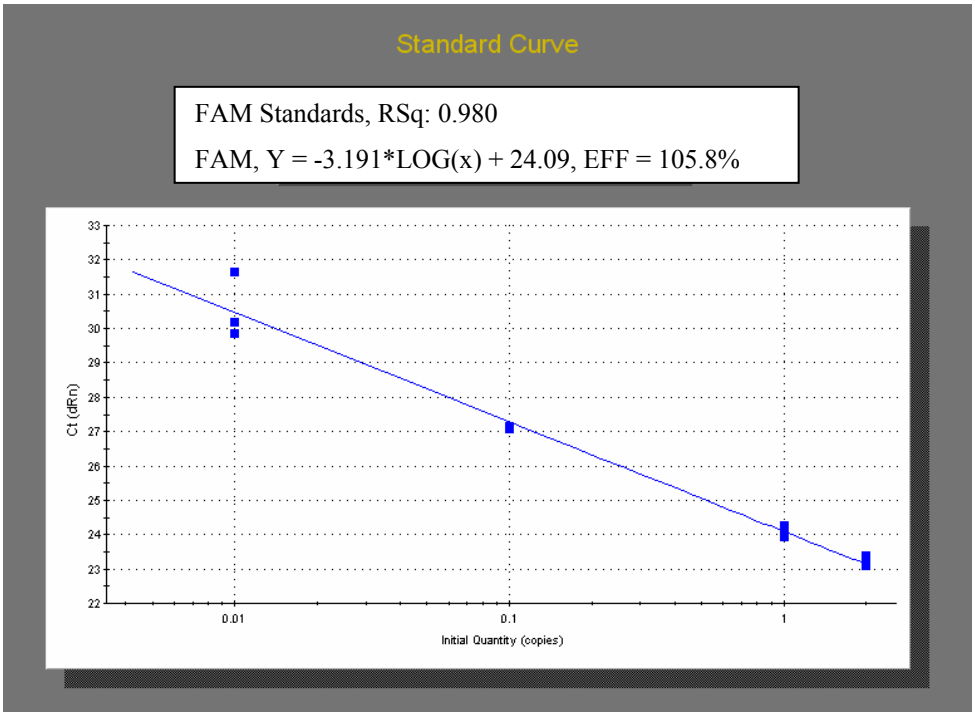
Standard curve for TGFβI



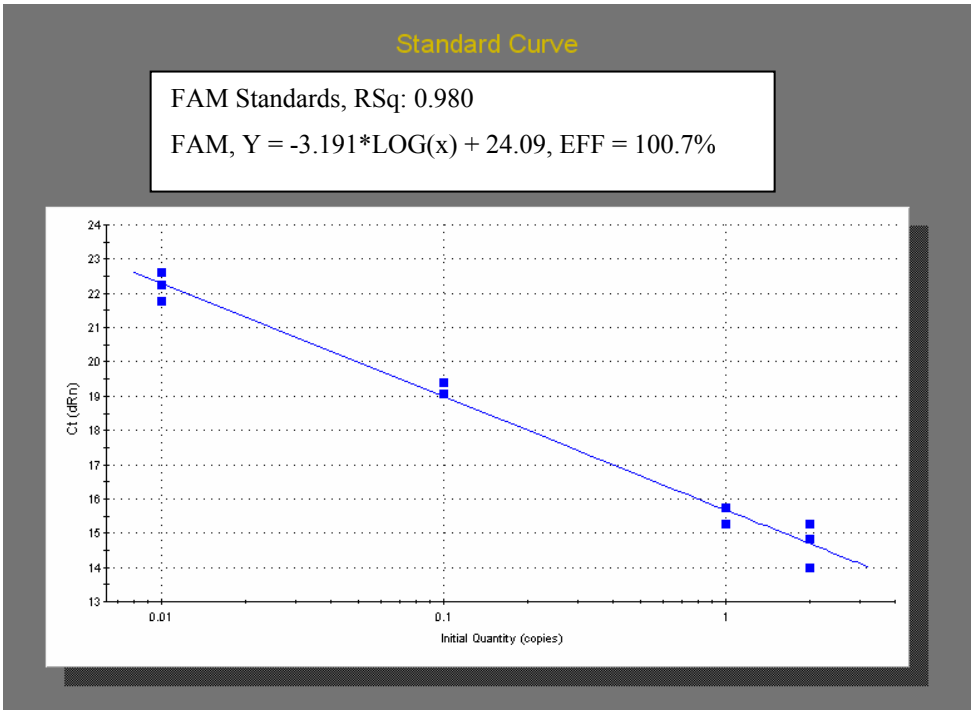
Standard curve for HPRT



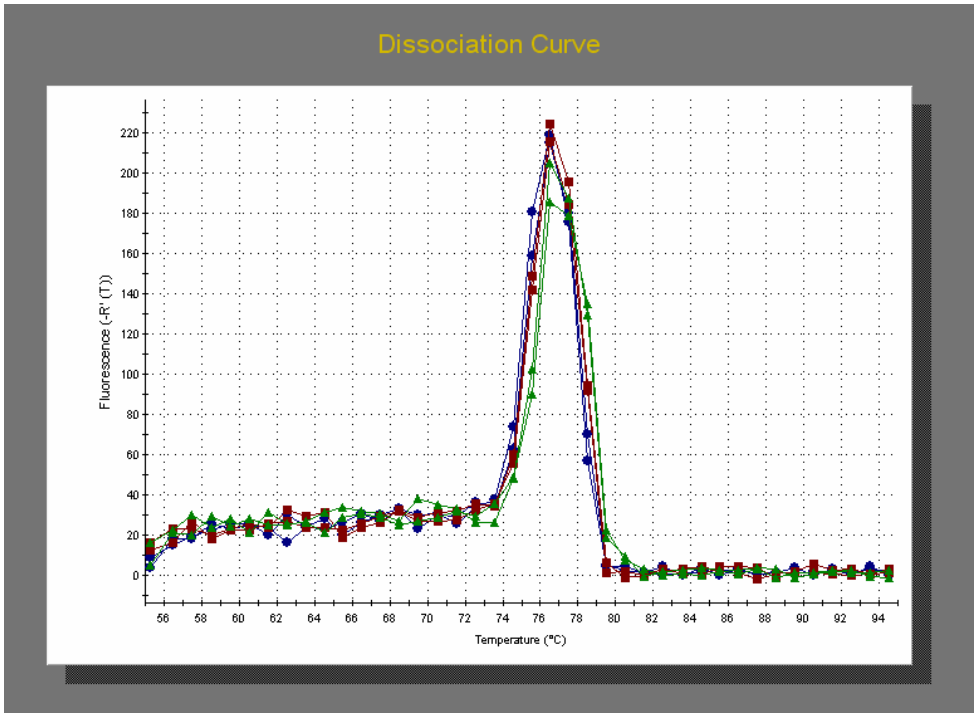
Standard curve for TFRC



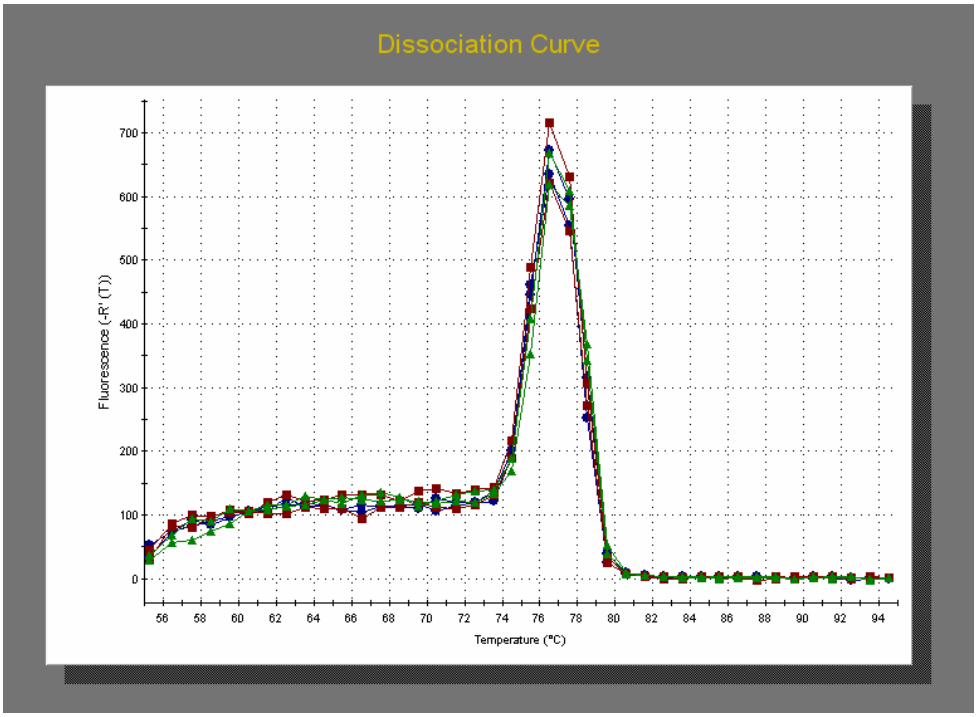
Standard curve for PBGD



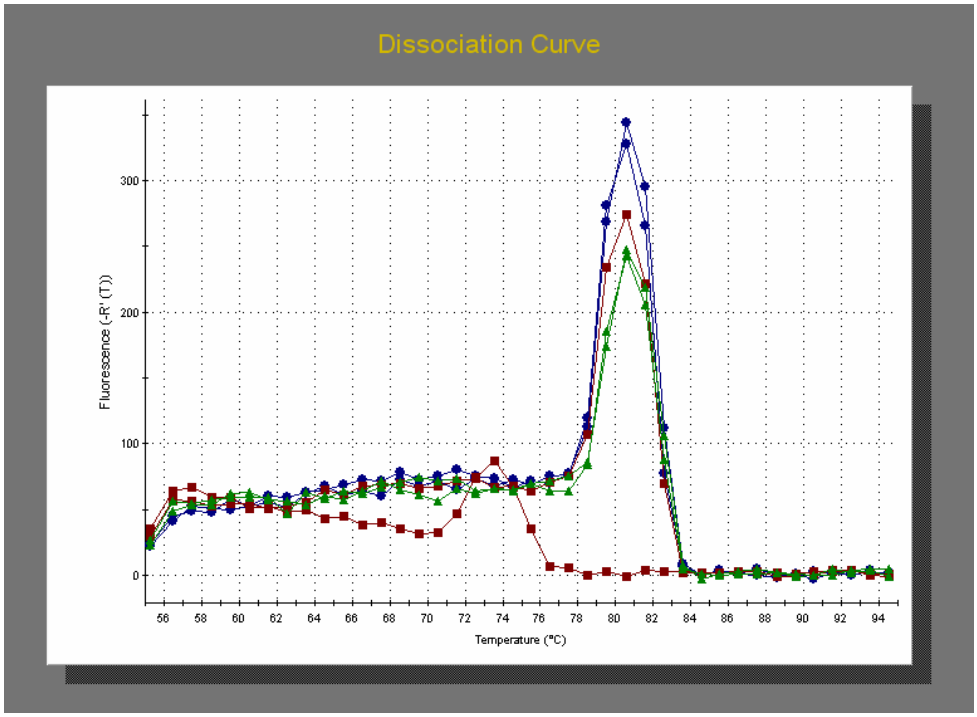
Dissociation curve for AKAP1 PCR products



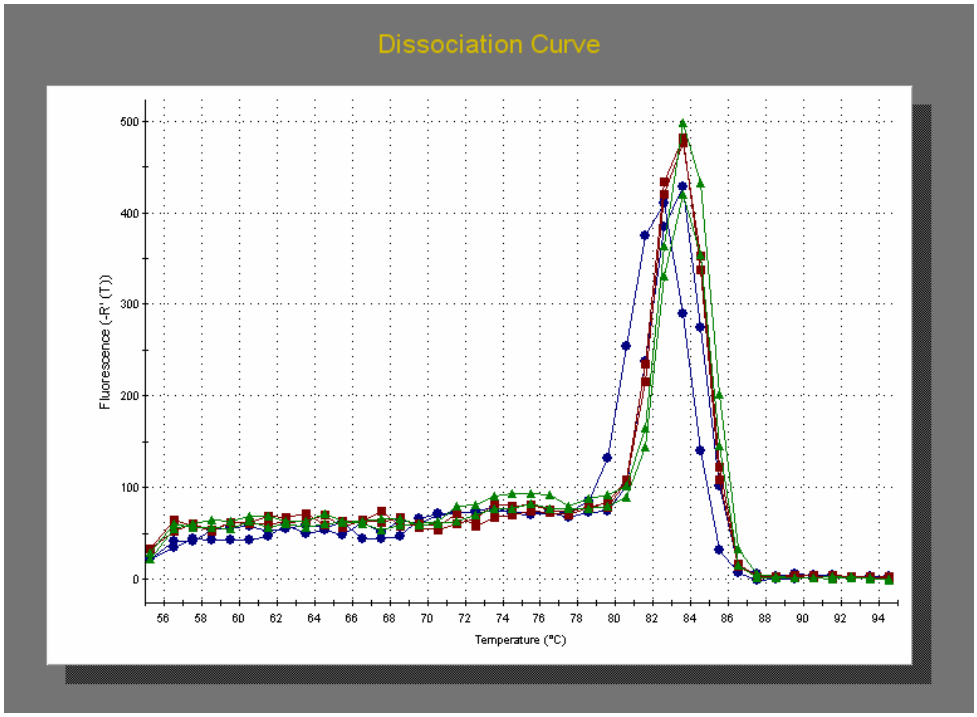
Dissociation curve for APRIL PCR products



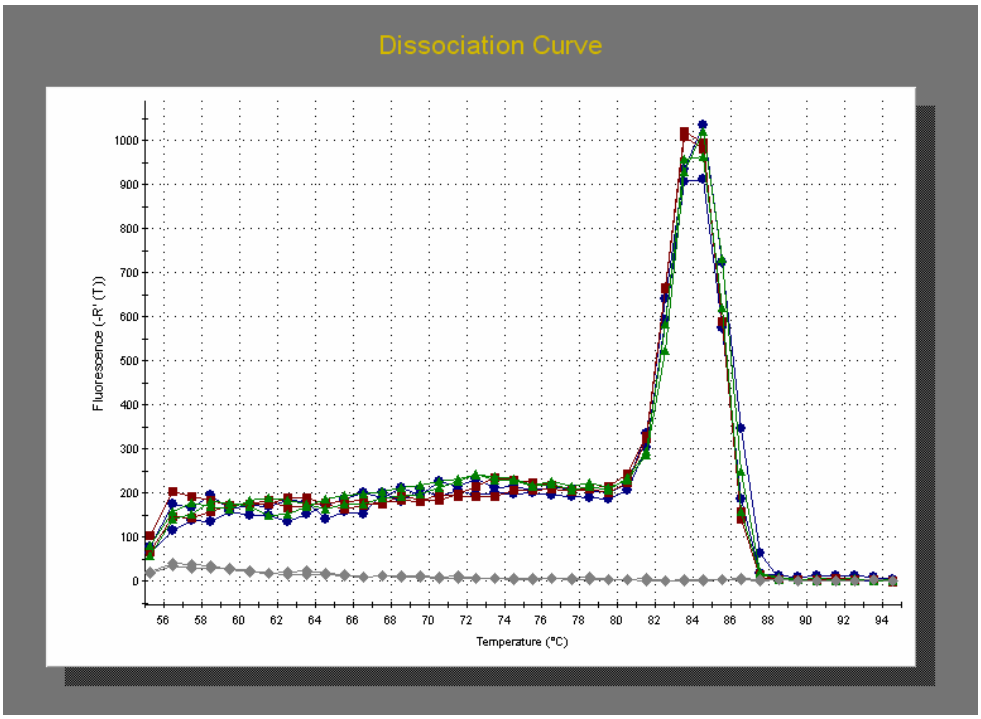
Dissociation curve for C/EBPα PCR products



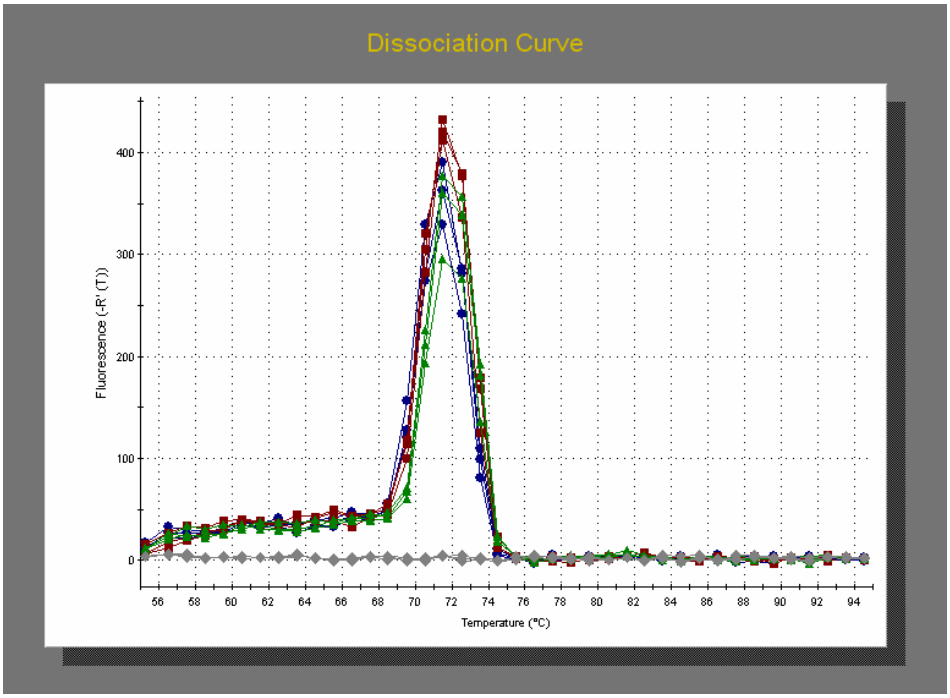
Dissociation curve for DDB2 PCR products



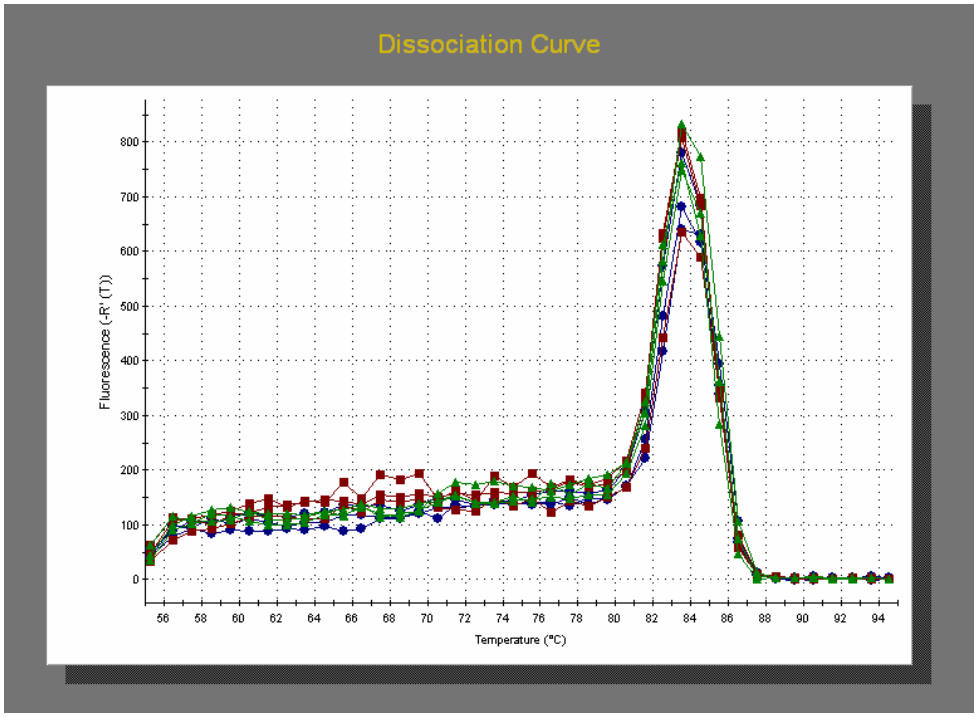
Dissociation curve for Granulin PCR products



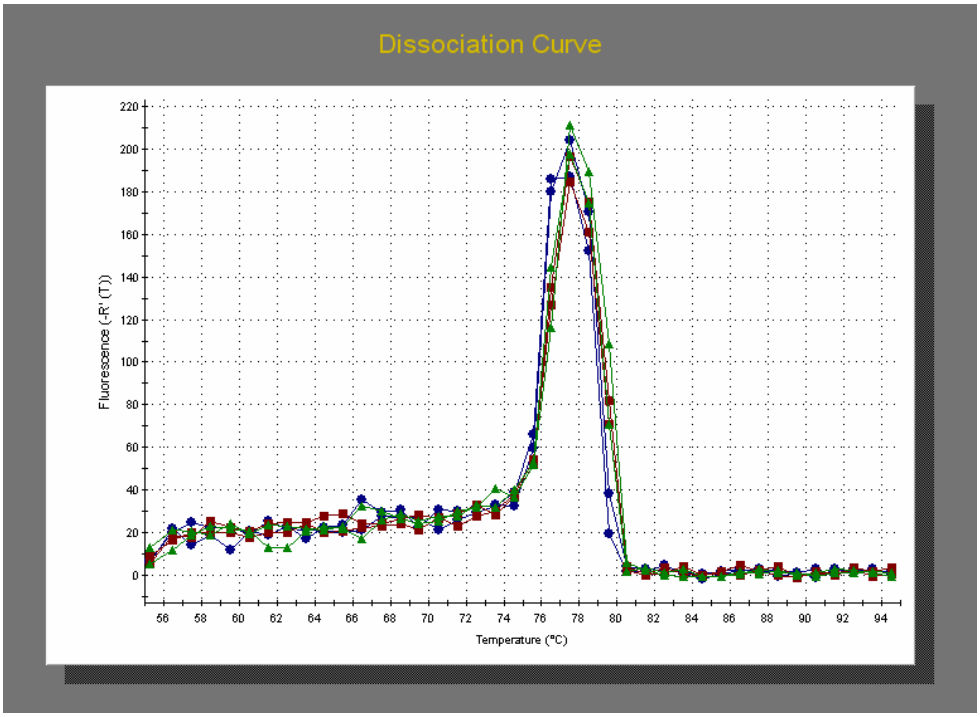
Dissociation curve for NCOA3 PCR products



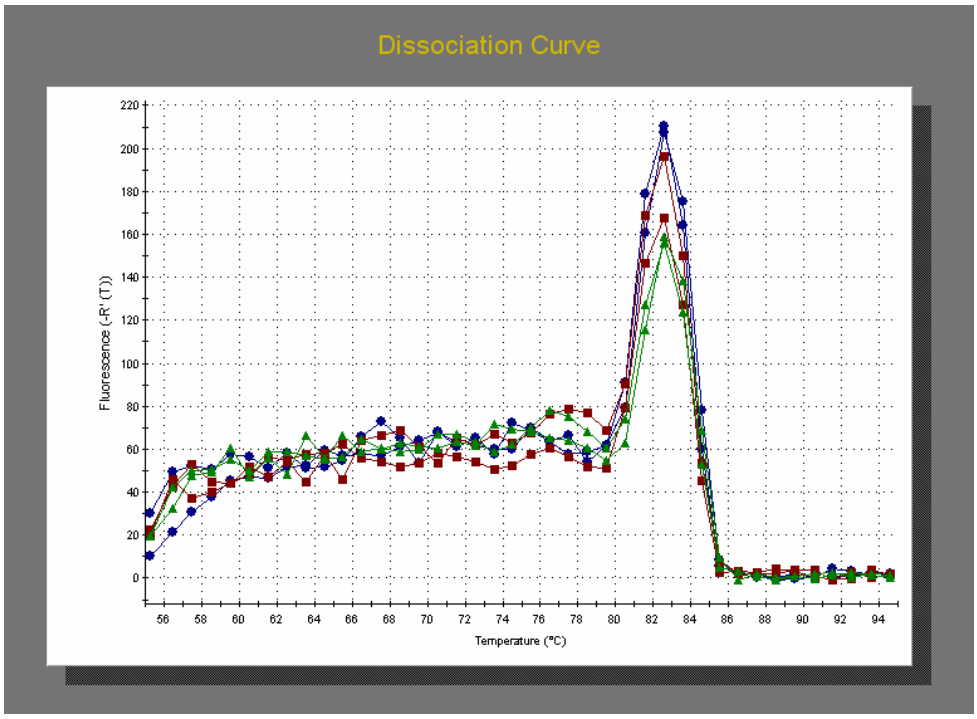
Dissociation curve for RARRES3 PCR products



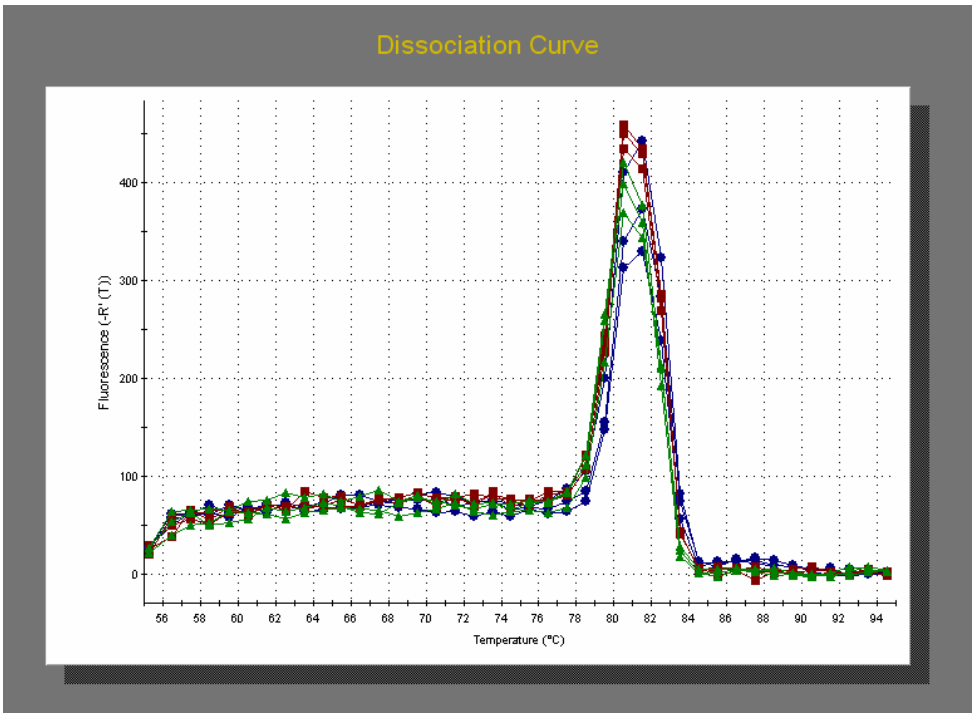
Dissociation curve for RBBP4 PCR products



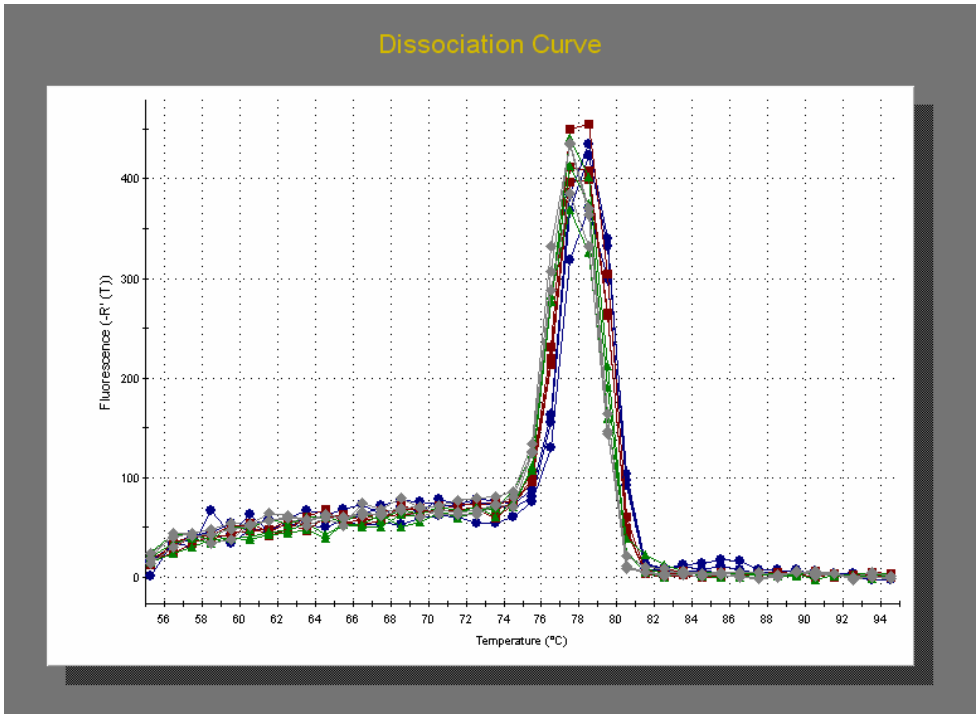
Dissociation curve for TGFβI PCR products



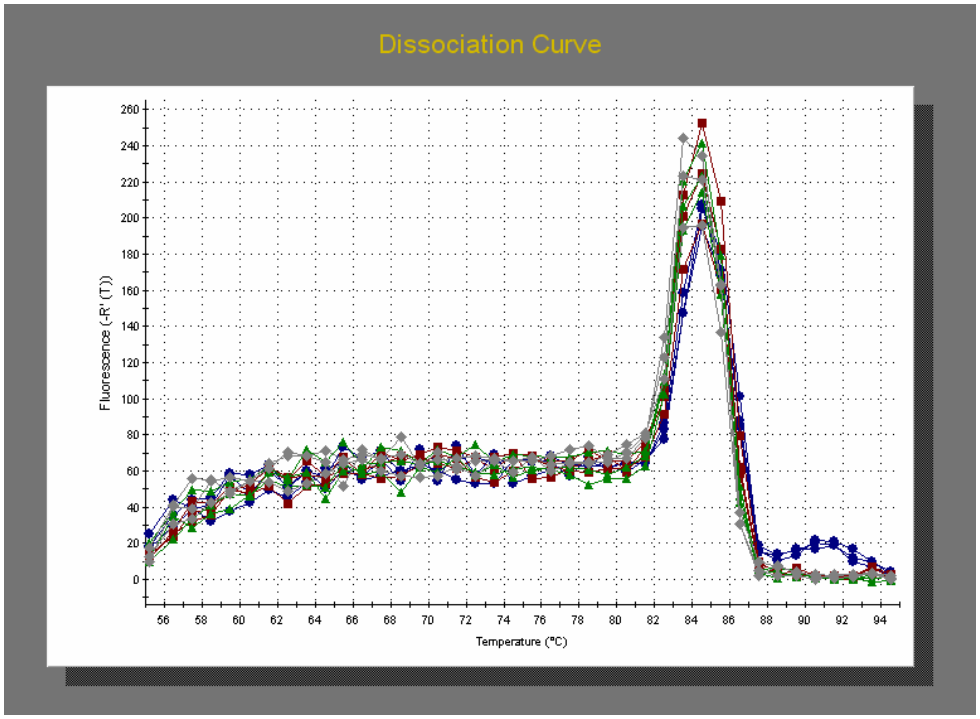
Dissociation curve for HPRT PCR products



Dissociation curve for TFRC PCR products



Dissociation curve for PBGD PCR products



Appendix III Raw Ct values for all RT-qPCR experiments

Relative Transcript Amount = 1/(Efficiency Gene) $^{Ct\ Gene}$

Combined Deviation = 1/√n X √(Standard deviation of target gene)^2 + (Standard deviation of HK genes)^2

Standard Error : Standard error of the mean (Combined deviation/√n

HPRT

Efficiency Value: **99.2**

Samples	CT 1	CT 2	CT 3	Mean Ct	Mean Relative Transcript Quantity
HBL-100	24.98	24.19	24.15	24.44	24.44
MDA-MB-231	26.08	26.74	26.71	26.51	26.51
MDA-MB-468	25.31	25.11	25.29	25.24	25.24
MCF-7	24.76	24.84	25.06	24.89	24.89
ZR-75-1	25.01	24.84	24.92	24.92	24.92
T-47D	27.26	27.13	27.23	27.21	27.21

TFRC

Efficiency Value: **105.8**

Samples	CT 1	CT 2	CT 3	Mean Ct	Mean Relative Transcript Quantity
HBL-100	24.98	24.19	24.15	24.44	24.44
MDA-MB-231	26.08	26.87	26.74	26.56	26.56
MDA-MB-468	25.31	25.11	25.29	25.24	25.24
MCF-7	24.76	24.84	25.06	24.89	24.89
ZR-75-1	25.01	24.84	24.92	24.92	24.92
T-47D	27.26	27.13	27.23	27.21	27.21

PBGD

Efficiency Value: **100.7**

Samples	CT 1	CT 2	CT 3	Mean Ct	Mean Relative Transcript Quantity
HBL-100	25.98	26.17	25.54	25.90	25.90
MDA-MB-231	26.12	25.68	26.31	26.04	26.04
MDA-MB-468	26.91	26.51	26.34	26.59	26.59
MCF-7	26.01	26.54	26.28	26.28	26.28
ZR-75-1	25.73	25.48	26.06	25.76	25.76
T-47D	25.1	25.29	25.11	25.17	25.17

NCOA3 expression relative to HKs

Efficiency Value: **104.8**

Samples	CT 1	CT 2	CT 3	Mean Ct	Mean NCOA3 Relative Transcript Quantity	Mean HK Relative Transcript Quantity	Mean NCOA3/HK Ratio	NCOA3 Standard Deviation	HK Standard Deviation	Combined Deviation	Standard Error
HBL-100	23.02	23.22	23.18	23.14	6.26135E-08	7.00497E-08	7.71	0.11	0.43	0.25	0.15
MDA-MB-231	28.04	28.25	28.02	28.10	1.78557E-09	1.92633E-08	9.37	0.13	0.38	0.23	0.13
MDA-MB-468	25.28	25.55	25.19	25.34	1.29854E-08	4.05595E-08	8.45	0.19	0.19	0.15	0.09
MCF-7	21.89	22	22.11	22.00	1.41788E-07	5.10465E-08	7.33	0.11	0.20	0.13	0.08
ZR-75-1	21.99	22.01	21.98	21.99	1.42178E-07	5.05151E-08	7.33	0.02	0.18	0.11	0.06
T-47D	24.57	24.44	24.49	24.50	2.35904E-08	1.44448E-08	8.17	0.07	0.08	0.06	0.04

RARRES3 expression relative to HKs

Efficiency Value: **101.1**

Samples	CT 1	CT 2	CT 3	Mean Ct	Mean RARRES3 Relative Transcript Quantity	Mean HK Relative Transcript Quantity	Mean RARRES3/HK Ratio	RARRES3 Standard Deviation	HK Standard Deviation	Combined Deviation	Standard Error
HBL-100	29.66	29.3	28.9	29.29	1.35078E-09	7.00497E-08	9.76	0.38	0.43	0.33	0.19
MDA-MB-231	27.59	27.49	27.03	27.37	5.10521E-09	1.92633E-08	9.12	0.30	0.38	0.28	0.16
MDA-MB-468	22.87	22.64	22.73	22.75	1.27128E-07	4.05595E-08	7.58	0.12	0.19	0.13	0.07
MCF-7	35.01	35.01	35.32	35.11	2.26975E-11	5.10465E-08	11.70	0.18	0.20	0.15	0.09
ZR-75-1	34.11	33.93	34.1	34.05	4.76339E-11	5.05151E-08	11.35	0.10	0.18	0.12	0.07
T-47D	28.6	28.28	28.89	28.59	2.17867E-09	1.44448E-08	9.53	0.31	0.08	0.18	0.11

AKAP1 expression relative to HKs

Efficiency Value: **82.7**

Samples	CT 1	CT 2	CT 3	Mean Ct	Mean AKAP1 Relative Transcript Quantity	Mean HK Relative Transcript Quantity	Mean AKAP1/HK Ratio	AKAP1 Standard Deviation	HK Standard Deviation	Combined Deviation	Standard Error
HBL-100	28	27.67	27.75	27.81	5.45353E-08	7.00497E-08	9.27	0.17	0.43	0.26	0.15
MDA-MB-231	28	28.35	27.88	28.08	4.65226E-08	1.92633E-08	9.36	0.24	0.38	0.26	0.15
MDA-MB-468	33	32.76	32.65	32.80	2.7E-09	4.05595E-08	10.93	0.18	0.19	0.15	0.09
MCF-7	26.98	26.76	26.85	26.86	9.59946E-08	5.10465E-08	8.95	0.11	0.20	0.13	0.08
ZR-75-1	25.89	25.66	25.71	25.75	1.87227E-07	5.05151E-08	8.58	0.12	0.18	0.13	0.07
T-47D	25.76	25.34	25.7	25.60	2.06269E-07	1.44448E-08	8.53	0.23	0.08	0.14	0.08

APRIL expression relative to HKs

Efficiency Value: **96**

Samples	CT 1	CT 2	CT 3	Mean Ct	Mean APRIL Relative Transcript Quantity	Mean HK Relative Transcript Quantity	Mean APRIL/HK Ratio	APRIL Standard Deviation	HK Standard Deviation	Combined Deviation	Standard Error
HBL-100	27.04	27.33	27.65	27.34	1.037E-08	7.00497E-08	9.11	0.31	0.43	0.30	0.17
MDA-MB-231	24.19	24.01	24.44	24.21	8.44373E-08	1.92633E-08	8.07	0.22	0.38	0.25	0.14
MDA-MB-468	23.12	23.45	23.12	23.23	1.63384E-07	4.05595E-08	7.74	0.19	0.19	0.16	0.09
MCF-7	33.65	32.78	32.54	32.99	2.39456E-10	5.10465E-08	11.00	0.58	0.20	0.36	0.21
ZR-75-1	28.58	28.87	28.43	28.63	4.33419E-09	5.05151E-08	9.54	0.22	0.18	0.17	0.10
T-47D	32.38	32.43	32.66	32.49	3.20654E-10	1.44448E-08	10.83	0.15	0.08	0.10	0.06

DDB2 expression relative to HKs

Efficiency Value: 87.2

Samples	CT 1	CT 2	CT 3	Mean Ct	Mean DDB2 Relative Transcript Quantity	Mean HK Relative Transcript Quantity	Mean DDB2/HK Ratio	DDB2 Standard Deviation	HK Standard Deviation	Combined Deviation	Standard Error
HBL-100	26.16	26.82	26.16	26.38	6.67455E-08	7.00497E-08	8.79	0.38	0.43	0.33	0.19
MDA-MB-231	28.22	28.3	28.5	28.34	1.92319E-08	1.92633E-08	9.45	0.14	0.38	0.23	0.13
MDA-MB-468	27.19	27.05	27.03	27.09	4.204E-08	4.05595E-08	9.03	0.09	0.19	0.12	0.07
MCF-7	23.07	23.02	23.17	23.09	5.17245E-07	5.10465E-08	7.70	0.08	0.20	0.12	0.07
ZR-75-1	23.88	23.55	23.34	23.59	3.80593E-07	5.05151E-08	7.86	0.27	0.18	0.19	0.11
T-47D	26.57	26.38	26.51	26.49	6.13856E-08	1.44448E-08	8.83	0.10	0.08	0.07	0.04

CEBP expression relative to HKs

Efficiency Value: **98.4**

Samples	CT 1	CT 2	CT 3	Mean Ct	Mean CEBP Relative Transcript Quantity	Mean HK Relative Transcript Quantity	Mean CEBP/HK Ratio	CEBP Standard Deviation	HK Standard Deviation	Combined Deviation	Standard Error
HBL-100	23.02	23.47	23.15	23.21	1.24919E-07	7.00497E-08	7.74	0.23	0.43	0.28	0.16
MDA-MB-231	26.89	26.42	26.03	26.45	1.39127E-08	1.92633E-08	8.82	0.43	0.38	0.33	0.19
MDA-MB-468	29.12	29.73	29.43	29.43	1.78093E-09	4.05595E-08	9.81	0.31	0.19	0.21	0.12
MCF-7	27.73	27.71	28.35	27.93	4.991E-09	5.10465E-08	9.31	0.36	0.20	0.24	0.14
ZR-75-1	29.17	29.34	29.01	29.17	2.09683E-09	5.05151E-08	9.72	0.17	0.18	0.14	0.08
T-47D	27.88	27.49	27.24	27.54	6.51067E-09	1.44448E-08	9.18	0.32	0.08	0.19	0.11

TGFBI expression relative to HKs

Efficiency Value: **100.2**

Samples	CT 1	CT 2	CT 3	Mean Ct	Mean TGFBI Relative Transcript Quantity	Mean HK Relative Transcript Quantity	Mean TGFBI/HK Ratio	TGFbI Standard Deviation	HK Standard Deviation	Combined Deviation	Standard Error
HBL-100	21.04	21.21	21.71	21.32	3.89181E-07	7.00497E-08	7.11	0.35	0.43	0.32	0.18
MDA-MB-231	24.49	24.35	24.55	24.46	4.33053E-08	1.92633E-08	8.15	0.10	0.38	0.22	0.13
MDA-MB-468	30.22	30.08	30.93	30.41	7.23166E-10	4.05595E-08	10.14	0.46	0.19	0.29	0.16
MCF-7	24.65	24.09	24.29	24.34	4.75785E-08	5.10465E-08	8.11	0.28	0.20	0.20	0.12
ZR-75-1	30.58	30.94	30.68	30.73	5.63252E-10	5.05151E-08	10.24	0.19	0.18	0.15	0.09
T-47D	28.38	28.45	28.97	28.60	2.49717E-09	1.44448E-08	9.53	0.32	0.08	0.19	0.11

GRANULIN expression relative to HKs

Efficiency Value: **109**

Samples	CT 1	CT 2	CT 3	Mean Ct	Mean Granulin Relative Transcript Quantity	Mean HK Relative Transcript Quantity	Mean GRANULIN/HK Ratio	Granulin Standard Deviation	HK Standard Deviation	Combined Deviation	Standard Error
HBL-100	23.04	23.31	23.73	23.36	3.3937E-08	7.00497E-08	7.79	0.35	0.43	0.32	0.18
MDA-MB-231	21.19	21.4	21.88	21.49	1.34754E-07	1.92633E-08	7.16	0.35	0.38	0.30	0.17
MDA-MB-468	22.55	22.08	22.93	22.52	6.37684E-08	4.05595E-08	7.51	0.43	0.19	0.27	0.16
MCF-7	19.3	19.94	19.68	19.64	5.25479E-07	5.10465E-08	6.55	0.32	0.20	0.22	0.13
ZR-75-1	21.76	21.12	21.17	21.35	1.49384E-07	5.05151E-08	7.12	0.36	0.18	0.23	0.13
T-47D	20.36	20.45	20.97	20.59	2.60165E-07	1.44448E-08	6.86	0.33	0.08	0.20	0.11

RBBP4 expression relative to HKs

Efficiency Value: **96**

Samples	CT 1	CT 2	CT 3	Mean Ct	Mean RBBP4 Relative Transcript Quantity	Mean HK Relative Transcript Quantity	Mean RBBP4/HK Ratio	RBBP4 Standard Deviation	HK Standard Deviation	Combined Deviation	Standard Error
HBL-100	27.04	27.21	27.73	27.33	9.67278E-09	7.00497E-08	9.11	0.36	0.43	0.32	0.19
MDA-MB-231	25.19	25.35	25.88	25.47	3.38628E-08	1.92633E-08	8.49	0.36	0.38	0.30	0.17
MDA-MB-468	23.11	23.08	23.93	23.37	1.42001E-07	4.05595E-08	7.79	0.48	0.19	0.30	0.17
MCF-7	28.65	28.09	29.17	28.64	4.09185E-09	5.10465E-08	9.55	0.54	0.20	0.33	0.19
ZR-75-1	23.58	23.94	23.68	23.73	1.08275E-07	5.05151E-08	7.91	0.19	0.18	0.15	0.09
T-47D	24.38	24.45	24.97	24.60	6.08726E-08	1.44448E-08	8.20	0.32	0.08	0.19	0.11

Formalin Fixed paraffin embedded tissues

FFPE Tissues

	HPRT	Efficiency Value:	99.2	1.992		
		CT		**Mean Ct**	**HPRT Relative Quantity**	**Standard Deviation**
Samples	1	2	3			
2047	34.01	34.20	34.35	34.19	1.92463E-11	0.17
2139	32.95	32.88	33.13	32.99	4.57596E-11	0.129
2140	33.05	33.88	34	33.64	2.84874E-11	0.517
2149	33	33.36	32.86	33.07	4.2985E-11	0.258
2185	31.41	30.99	31.88	31.43	1.41077E-10	0.445
2182	30.47	29.93	30.3	30.23	3.33812E-10	0.276
2183	30.2	31.41	31.12	30.91	2.04835E-10	0.632
2093	32.41	32.58	32.6	32.53	6.36242E-11	0.104
2095	34.45	33.69	33.97	34.04	2.14469E-11	0.384
2096	34.11	33.69	34.22	34.01	2.19163E-11	0.280
2070	31.28	31.56	30.97	31.27	1.57966E-10	0.295
2074	32.56	33.44	33.02	33.01	4.51038E-11	0.440
2076	31.12	31.11	31.66	31.30	1.54955E-10	0.315
2215	34.3	34.33	34.1	34.24	1.84751E-11	0.125
2205	26.55	26.21	26.47	26.41	5.27118E-09	0.178
2204	29.99	29.25	30.63	29.96	4.07588E-10	0.691
2201	32.43	32.4	32.81	32.55	6.28634E-11	0.229
2200	28.47	28.19	28.75	28.47	1.19182E-09	0.280
2196	32.49	32.54	32.88	32.64	5.89099E-11	0.212
2195	31.41	31.5	31.68	31.53	1.30939E-10	0.137
2137	33.12	32.55	32.41	32.69	5.65491E-11	0.376
2105	33.14	33.02	33.29	33.15	4.06711E-11	0.135
2019	33.18	33.61	33.67	33.49	3.18977E-11	0.267

	TFRC	Efficiency Value:		**105.8**	2.058	
		CT		**Mean Ct**	**TFRC Relative Quantity**	**Standard Deviation**
Samples	1	2	3			
2047	30.32	30.60	30.32	30.41	2.93144E-10	0.16
2139	32.00	32.08	32.86	32.31	7.43935E-11	0.48
2140	32.15	31.84	32.06	32.02	9.21561E-11	0.16
2149	31.91	32.08	32.19	32.06	8.93185E-11	0.14
2185	31.57	31.08	31.41	31.35	1.48745E-10	0.25
2182	27.37	26.88	26.77	27.01	3.42677E-09	0.32
2183	28.28	28.01	27.74	28.01	1.6611E-09	0.27
2093	34.58	34.61	34.02	34.40	1.64602E-11	0.33
2095	34.42	34.44	34.41	34.42	1.62243E-11	0.02
2096	32.13	32.66	32.81	32.53	6.34713E-11	0.36
2070	30.00	30.06	30.13	30.06	3.77387E-10	0.07
2074	33.33	33.17	33.01	33.17	4.00882E-11	0.16
2076	33.63	33.04	33.00	33.22	3.85745E-11	0.35
2215	29.86	29.74	29.96	29.85	4.39148E-10	0.11
2205	27.88	28.79	28.68	28.45	1.20915E-09	0.50
2204	27.80	27.71	27.70	27.74	2.02335E-09	0.06
2201	30.71	30.87	30.12	30.57	2.62434E-10	0.40
2200	30.29	30.01	30.87	30.39	2.98123E-10	0.44
2196	30.94	31.56	31.71	31.40	1.43473E-10	0.41
2195	29.92	28.85	28.56	29.11	7.5094E-10	0.72
2137	29.85	29.88	29.43	29.72	4.83508E-10	0.25
2105	29.99	29.48	30.02	29.83	4.46606E-10	0.30
2019	32.33	32.01	32.51	32.28	7.60219E-11	0.25
2105	29.99	29.48	30.02	29.83	4.46606E-10	0.30
2019	32.33	32.01	32.51	32.28	7.60219E-11	0.25

	PBGD	Efficiency Value:		100.7	2	
		CT		Mean Ct	PGBD Relative Quantity	Standard Deviation
Samples	1	2	3			
2047	31.57	31.45	31.66	31.56	1.28134E-10	0.105356538
2139	31.22	31.58	31.71	31.50	1.33483E-10	0.25
2140	31.24	31.25	31.83	31.44	1.39726E-10	0.34
2149	32.47	32.13	32.19	32.26	7.71272E-11	0.18
2185	31.82	31.81	31.36	31.66	1.18925E-10	0.26
2182	27.22	27.45	27.67	27.45	2.49442E-09	0.23
2183	31.00	31.23	31.00	31.08	1.8162E-10	0.13
2093	31.16	31.89	31.12	31.39	1.4486E-10	0.43
2095	31.56	31.87	31.60	31.68	1.17786E-10	0.17
2096	30.63	30.28	30.26	30.39	2.98123E-10	0.21
2070	30.75	30.74	30.02	30.50	2.74708E-10	0.42
2074	30.57	30.84	30.94	30.78	2.24443E-10	0.19
2076	31.77	30.07	30.79	30.88	2.09822E-10	0.85
2215	31.16	31.53	31.19	31.29	1.55328E-10	0.21
2205	30.49	30.74	30.13	30.45	2.84802E-10	0.31
2204	30.19	30.16	30.42	30.26	3.28237E-10	0.14
2201	29.64	29.09	29.65	29.46	5.8331E-10	0.32
2200	28.60	28.41	28.45	28.49	1.17757E-09	0.10
2196	30.40	29.56	29.97	29.98	4.01747E-10	0.42
2195	26.43	26.05	27.41	26.63	4.49728E-09	0.70
2137	28.08	28.79	28.98	28.62	1.07211E-09	0.47
2105	30.04	30.74	30.29	30.36	3.05382E-10	0.35
2019	32.09	31.38	31.75	31.74	1.12524E-10	0.36

AKAP1 expression relative to HKs

	Efficiency Value:	**82.7**	**1.827**

Samples	CT 1.00	CT 2.00	CT 3.00	**Mean Ct**	**AKAP1 Relative Transcript Quantity**	**AKAP1 Standard Deviation**	**Mean HK Quantity**	**Ratio AKAP1/HK**	**Standard Deviation of Ratios**	**Standard Error**
2047	33.39	33.50	33.07	33.29	2.0131E-09	0.30	1.46842E-10	13.71	0.18	0.10
2139	32.05	31.79	32.26	32.03	4.29592E-09	0.33	8.45454E-11	50.81	0.19	0.11
2140	33.87	33.70	36.13	34.92	7.55104E-10	1.72	8.67899E-11	8.70	0.99	0.57
2149	33.07	32.46	32.94	32.70	2.86223E-09	0.34	6.98102E-11	41.00	0.20	0.11
2185	32.45	32.33	33.82	33.08	2.28418E-09	1.05	1.36249E-10	16.76	0.61	0.35
2182	30.37	31.60	30.89	31.25	6.86818E-09	0.50	2.085E-09	3.29	0.29	0.17
2183	31.64	32.04	31.79	31.92	4.58982E-09	0.18	6.82517E-10	6.72	0.10	0.06
2093	37.67	36.71	36.50	36.61	2.73195E-10	0.15	7.49816E-11	3.64	0.09	0.05
2095	33.34	33.18	33.78	33.48	1.79027E-09	0.42	5.18192E-11	34.55	0.24	0.14
2096	33.46	33.38	33.96	33.67	1.59691E-09	0.41	1.27837E-10	12.49	0.24	0.14
2070	32.03	32.46	32.81	32.64	2.97637E-09	0.25	2.7002E-10	11.02	0.14	0.08
2074	33.63	34.83	33.49	34.16	1.18922E-09	0.95	1.03212E-10	11.52	0.55	0.32
2076	32.90	32.09	32.03	32.06	4.20642E-09	0.04	1.34451E-10	31.29	0.02	0.01
2215	34.59	34.99	34.33	34.66	8.80296E-10	0.47	2.04317E-10	4.31	0.27	0.16
2205	32.29	32.06	32.57	32.32	3.6082E-09	0.36	2.25504E-09	1.60	0.21	0.12
2204	32.10	31.62	31.79	31.71	5.20788E-09	0.12	9.19724E-10	5.66	0.07	0.04
2201	32.40	32.59	33.71	33.15	2.18341E-09	0.79	3.02869E-10	7.21	0.46	0.26
2200	32.29	31.57	32.16	31.87	4.72997E-09	0.42	8.8917E-10	5.32	0.24	0.14
2196	32.65	31.63	32.55	32.09	4.13118E-09	0.65	2.01377E-10	20.51	0.38	0.22
2195	34.01	34.30	34.29	34.30	1.09645E-09	0.01	1.79305E-09	0.61	0.00	0.00
2137	31.42	34.28	34.08	34.18	1.17499E-09	0.14	5.37388E-10	2.19	0.08	0.05
2105	32.70	31.59	32.64	32.12	4.06952E-09	0.74	2.6422E-10	15.40	0.43	0.25
2019	32.63	32.06	32.57	32.32	3.6082E-09	0.36	7.34811E-11	49.10	0.21	0.12

APRIL expression relative to HKs

	Efficiency Value:	**96**	**1.96**

Samples	CT 1	CT 2	CT 3	**Mean Ct**	**APRIL Transcript Relative Quantity**	**APRIL Standard Deviation**	**Mean HK Quantity**	**Ratio APRIL/HK**	**Standard Deviation of Ratios**	**Standard Error**
2047	33.24	33.26	33.49	33.33	1.77966E-10	0.12	1.46842E-10	1.21	0.07	0.04
2139	33.67	33.74	33.00	33.47	1.72851E-10	0.37	8.45454E-11	2.04	0.22	0.12
2140	35.98	37.80	37.04	36.94	1.28985E-11	0.47	8.67899E-11	0.15	0.27	0.16
2149	35.06	36.20	36.11	35.79	2.94469E-11	0.22	6.98102E-11	0.42	0.12	0.07
2185	35.35	34.84	35.33	35.17	5.465E-11	0.25	1.36249E-10	0.40	0.14	0.08
2182	30.05	29.64	30.09	29.93	1.84402E-09	0.23	2.085E-09	0.88	0.13	0.08
2183	30.55	30.61	30.73	30.63	1.0975E-09	0.06	6.82517E-10	1.61	0.04	0.02
2093	37.92	37.51	37.84	37.76	9.57848E-12	0.17	7.49816E-11	0.13	0.10	0.06
2095	33.46	33.09	32.94	33.16	2.17128E-10	0.11	5.18192E-11	4.19	0.07	0.04
2096	35.49	34.96	36.06	35.50	4.19408E-11	0.55	1.27837E-10	0.33	0.32	0.18
2070	31.34	32.05	31.77	31.72	4.92741E-10	0.18	2.7002E-10	1.82	0.10	0.06
2074	34.51	34.86	35.09	34.82	6.21504E-11	0.15	1.03212E-10	0.60	0.08	0.05
2076	33.42	33.73	33.51	33.55	1.51651E-10	0.12	1.34451E-10	1.13	0.07	0.04
2215	32.32	32.02	31.87	32.07	4.48438E-10	0.10	2.04317E-10	2.19	0.06	0.03
2205	32.06	31.78	31.55	31.80	5.40611E-10	0.14	2.25504E-09	0.24	0.08	0.05
2204	29.96	29.57	29.66	29.73	2.15592E-09	0.08	9.19724E-10	2.34	0.05	0.03
2201	32.37	32.48	32.35	32.40	3.37272E-10	0.07	3.02869E-10	1.11	0.04	0.02
2200	32.35	32.37	32.27	32.33	3.57528E-10	0.05	8.8917E-10	0.40	0.03	0.02
2196	32.62	33.29	33.99	33.30	1.59085E-10	0.40	2.01377E-10	0.79	0.23	0.13
2195	32.68	33.30	33.11	33.03	2.0544E-10	0.14	1.79305E-09	0.11	0.08	0.05
2137	33.35	34.48	34.33	34.05	9.5321E-11	0.22	5.37388E-10	0.18	0.12	0.07
2105	34.79	33.66	34.79	34.41	9.5321E-11	0.58	2.6422E-10	0.36	0.33	0.19
2019	34.94	34.36	34.94	34.75	7.30988E-11	0.30	7.34811E-11	0.99	0.17	0.10

CEBP alpha expression relative to HKs

	Efficiency Value:	**98.4**	1.985

Samples	CT 1	CT 2	CT 3	Mean Ct	C/EBPalpha Transcript Relative Quantity	C/EBP alpha Standard Deviation	Mean HK Quantity	Ratio C/EBPalpha/HK	Standard Deviation of Ratios	Standard Error
2047	33.23	33.37	33.75	33.45	1.0425E-10	0.20	1.46842E-10	0.71	0.12	0.07
2139	36.10	36.76	36.86	36.57	1.15591E-11	0.15	8.45454E-11	0.14	0.08	0.05
2140	30.29	33.18	31.21	31.56	2.99658E-10	1.05	8.67899E-11	3.45	0.61	0.35
2149	31.56	31.40	31.76	31.57	3.95717E-10	0.18	6.98102E-11	5.67	0.10	0.06
2185	34.20	34.89	34.48	34.52	4.87779E-11	0.23	1.36249E-10	0.36	0.13	0.08
2182	29.08	30.20	30.79	30.02	9.25993E-10	0.40	2.085E-09	0.44	0.23	0.13
2183	29.18	30.27	28.95	29.47	1.57593E-09	0.67	6.82517E-10	2.31	0.38	0.22
2093	35.68	35.97	35.33	35.66	2.42017E-11	0.32	7.49816E-11	0.32	0.18	0.11
2095	33.21	33.35	33.92	33.49	9.97438E-11	0.30	5.18192E-11	1.92	0.17	0.10
2096	35.21	35.85	36.40	35.82	1.87791E-11	0.33	1.27837E-10	0.15	0.19	0.11
2070	30.57	30.49	31.59	30.88	5.93012E-10	0.56	2.7002E-10	2.20	0.32	0.19
2074	32.76	32.02	32.24	32.34	2.58292E-10	0.16	1.03212E-10	2.50	0.09	0.05
2076	32.11	32.13	32.25	32.16	2.61659E-10	0.06	1.34451E-10	1.95	0.04	0.02
2215	32.11	32.19	32.86	32.39	2.13339E-10	0.34	2.04317E-10	1.04	0.20	0.11
2205	33.34	33.18	33.65	33.39	1.12931E-10	0.24	2.25504E-09	0.05	0.14	0.08
2204	30.38	29.21	29.59	29.73	1.63463E-09	0.27	9.19724E-10	1.78	0.15	0.09
2201	32.17	32.25	32.86	32.43	2.08518E-10	0.31	3.02869E-10	0.69	0.18	0.10
2200	33.03	33.04	31.17	32.41	2.56919E-10	0.95	8.8917E-10	0.29	0.55	0.32
2196	34.21	34.85	34.33	34.46	5.16459E-11	0.27	2.01377E-10	0.26	0.16	0.09
2195	30.41	30.67	30.38	30.49	8.21609E-10	0.15	1.79305E-09	0.46	0.08	0.05
2137	34.10	34.94	34.02	34.35	5.56916E-11	0.47	5.37388E-10	0.10	0.27	0.16
2105	34.97	34.53	34.23	34.58	5.53954E-11	0.19	2.6422E-10	0.21	0.11	0.06
2019	32.93	33.02	33.10	33.02	1.44657E-10	0.05	7.34811E-11	1.97	0.03	0.02

DBB2 expression relative to HKs

Efficiency Value:	**87.2**	**1.872**

Samples	CT 1	CT 2	CT 3	Mean Ct	DDB2 Transcript Relative Quantity	DDB2 Standard Deviation	Mean HK Quantity	Ratio DDB2/HK	Standard Deviation of Ratios	Standard Error
2047	35.26	32.07	37.07	34.80	3.81588E-10	2.50	1.46842E-10	2.60	1.45	0.83
2139	37.47	37.09	36.00	36.85	1.09046E-10	0.57	8.45454E-11	1.29	0.33	0.19
2140	36.84	36.03	37.35	36.74	1.051E-10	0.66	8.67899E-11	1.21	0.38	0.22
2149	37.49	35.99	38.90	37.46	6.59982E-11	1.46	6.98102E-11	0.95	0.84	0.49
2185	38.67	37.05	37.65	37.79	6.40982E-11	0.39	1.36249E-10	0.47	0.23	0.13
2182	37.08	36.75	36.54	36.79	1.0598E-10	0.13	2.085E-09	0.05	0.08	0.04
2183	32.97	33.77	34.12	33.62	6.33558E-10	0.26	6.82517E-10	0.93	0.15	0.09
2093	38.94	38.52	38.99	38.82	2.87866E-11	0.24	7.49816E-11	0.38	0.14	0.08
2095	35.14	35.80	35.76	35.57	1.96263E-10	0.12	5.18192E-11	3.79	0.07	0.04
2096	34.56	34.66	34.78	34.67	3.68546E-10	0.07	1.27837E-10	2.88	0.04	0.02
2070	33.51	33.82	33.73	33.69	6.7074E-10	0.07	2.7002E-10	2.48	0.04	0.02
2074	34.44	34.75	34.98	34.72	3.4283E-10	0.14	1.03212E-10	3.32	0.08	0.05
2076	36.54	36.06	36.32	36.31	1.41736E-10	0.15	1.34451E-10	1.05	0.08	0.05
2215	33.12	33.45	32.84	33.14	9.7851E-10	0.31	2.04317E-10	4.79	0.18	0.10
2205	33.31	34.23	34.68	34.07	4.65888E-10	0.31	2.25504E-09	0.21	0.18	0.10
2204	31.93	31.01	33.35	32.10	1.8184E-09	1.17	9.19724E-10	1.98	0.68	0.39
2201	34.04	34.40	34.55	34.33	4.37925E-10	0.11	3.02869E-10	1.45	0.06	0.04
2200	33.63	33.97	35.29	34.30	4.13361E-10	0.69	8.8917E-10	0.46	0.40	0.23
2196	34.39	34.94	35.63	34.99	2.72334E-10	0.39	2.01377E-10	1.35	0.22	0.13
2195	35.33	35.03	34.32	34.89	3.58186E-10	0.38	1.79305E-09	0.20	0.22	0.13
2137	38.33	38.30	38.39	38.34	3.773E-11	0.05	5.37388E-10	0.07	0.03	0.02
2105	36.04	35.55	36.15	35.91	1.77313E-10	0.30	2.6422E-10	0.67	0.17	0.10
2019	34.20	34.80	35.45	34.82	3.0165E-10	0.37	7.34811E-11	4.11	0.21	0.12

GRANULIN expression relative to HKs

Efficiency Value: **109** **2.019**

Samples	CT 1	CT 2	CT 3	Mean Ct	Granulin Transcript Relative Quantity	Granulin Standard Deviation	Mean HK Quantity	Ratio Granulin/HK	Standard Deviation of Ratios	Standard Error
2047	30.60	30.17	30.63	30.47	5.29201E-10	0.23	1.46842E-10	3.60	0.13	0.08
2139	31.55	30.78	31.11	31.15	3.49717E-10	0.20	8.45454E-11	4.14	0.12	0.07
2140	31.48	32.67	32.08	32.08	1.44046E-10	0.34	8.67899E-11	1.66	0.20	0.11
2149	32.67	32.08	32.77	32.51	1.27242E-10	0.35	6.98102E-11	1.82	0.20	0.12
2185	31.73	32.43	30.78	31.65	2.28419E-10	0.83	1.36249E-10	1.68	0.48	0.28
2182	26.76	27.03	26.35	26.71	7.2324E-09	0.34	2.085E-09	3.47	0.20	0.11
2183	29.52	29.90	29.39	29.60	9.22247E-10	0.26	6.82517E-10	1.35	0.15	0.09
2093	33.51	35.20	32.88	33.86	4.34951E-11	1.16	7.49816E-11	0.58	0.67	0.39
2095	31.86	32.37	33.03	32.42	1.14166E-10	0.37	5.18192E-11	2.20	0.21	0.12
2096	33.20	33.79	32.98	33.32	6.70617E-11	0.41	1.27837E-10	0.52	0.23	0.14
2070	30.08	29.98	30.50	30.19	6.08983E-10	0.26	2.7002E-10	2.26	0.15	0.09
2074	34.41	33.39	33.01	33.60	6.84893E-11	0.30	1.03212E-10	0.66	0.17	0.10
2076	32.72	32.46	32.70	32.63	1.1506E-10	0.12	1.34451E-10	0.86	0.07	0.04
2215	31.00	31.05	31.07	31.04	3.39767E-10	0.02	2.04317E-10	1.66	0.01	0.01
2205	29.38	28.61	28.63	28.87	1.7677E-09	0.15	2.25504E-09	0.78	0.08	0.05
2204	28.41	28.53	28.15	28.36	2.27072E-09	0.19	9.19724E-10	2.47	0.11	0.06
2201	30.36	30.09	29.78	30.08	7.20752E-10	0.18	3.02869E-10	2.38	0.10	0.06
2200	29.08	29.38	29.01	29.16	1.26393E-09	0.19	8.8917E-10	1.42	0.11	0.06
2196	31.15	31.88	30.72	31.25	2.89101E-10	0.58	2.01377E-10	1.44	0.34	0.19
2195	30.56	30.57	30.17	30.43	5.40888E-10	0.20	1.79305E-09	0.30	0.12	0.07
2137	32.39	31.91	31.94	32.08	1.7768E-10	0.09	5.37388E-10	0.33	0.05	0.03
2105	31.34	31.61	31.37	31.44	2.52997E-10	0.12	2.6422E-10	0.96	0.07	0.04
2019	30.95	31.33	31.14	31.14	3.05804E-10	0.11	7.34811E-11	4.16	0.06	0.04

NCOA3 expression relative to HKs

	Efficiency Value:	**104.8**	**2.048**

Samples	CT 1	CT 2	CT 3	**Mean Ct**	**NCOA3 Transcript Relative Quantity**	**NCOA3 Standard Deviation**	**Mean HK Quantity**	**Ratio NCOA3/HK**	**Standard Deviation of Ratios**	**Standard Error**
2047	30.21	30.16	31.16	30.51	3.17193E-10	0.56	1.46842E-10	2.16	0.34	0.19
2139	34.94	34.09	34.02	34.35	2.02216E-11	0.51	8.45454E-11	0.24	0.35	0.20
2140	31.40	32.05	32.67	32.04	1.05923E-10	0.64	8.67899E-11	1.22	0.42	0.24
2149	32.34	32.78	33.51	32.88	5.81445E-11	0.59	6.98102E-11	0.83	0.36	0.21
2185	34.45	34.38	34.01	34.28	2.12623E-11	0.24	1.36249E-10	0.16	0.24	0.14
2182	29.77	29.71	29.73	29.74	5.52185E-10	0.03	2.085E-09	0.26	0.16	0.01
2183	30.36	30.80	31.74	30.97	2.28638E-10	0.70	6.82517E-10	0.33	0.47	0.27
2093	34.61	34.05	34.05	34.24	2.19331E-11	0.32	7.49816E-11	0.29	0.26	0.15
2095	31.05	31.39	31.52	31.32	1.77479E-10	0.24	5.18192E-11	3.42	0.20	0.11
2096	35.68	34.07	34.74	34.83	1.43343E-11	0.81	1.27837E-10	0.11	0.50	0.03
2070	31.66	31.58	30.40	31.21	1.91582E-10	0.71	2.7002E-10	0.71	0.44	0.26
2074	33.74	34.62	33.54	33.97	2.6617E-11	0.57	1.03212E-10	0.26	0.37	0.21
2076	30.24	30.46	30.33	30.34	3.57447E-10	0.11	1.34451E-10	2.66	0.33	0.19
2215	28.76	28.79	28.91	28.82	1.0653E-09	0.08	2.04317E-10	5.21	0.10	0.06
2205	29.21	29.05	29.20	29.15	8.38869E-10	0.09	2.25504E-09	0.37	0.21	0.12
2204	31.90	31.18	31.72	31.60	1.45202E-10	0.37	9.19724E-10	0.16	0.32	0.02
2201	31.11	31.78	31.30	31.40	1.67988E-10	0.35	3.02869E-10	0.55	0.27	0.16
2200	31.73	30.59	31.27	31.20	1.93885E-10	0.57	8.8917E-10	0.22	0.38	0.02
2196	32.51	32.85	31.95	32.44	7.97066E-11	0.45	2.01377E-10	0.40	0.33	0.19
2195	31.08	32.31	32.02	31.80	1.25508E-10	0.64	1.79305E-09	0.07	0.50	0.03
2137	35.15	33.37	34.00	34.17	2.29519E-11	0.90	5.37388E-10	0.04	0.57	0.03
2105	32.23	32.85	33.00	32.69	6.63111E-11	0.41	2.6422E-10	0.25	0.29	0.17
2019	34.33	33.89	34.00	34.07	2.46576E-11	0.23	7.34811E-11	0.34	0.22	0.12

RARRES3 expression relative to HKs

Efficiency Value: **101** **2.01**

Samples	CT 1	CT 2	CT 3	Mean Ct	RARRES3 Transcript Relative Quantity	RARRES3 Standard Deviation	Mean HK Quantity	Ratio RARRES3/HK	Standard Deviation of Ratios	Standard Error
2047	32.16	32.45	31.97	32.19	2.0363E-10	0.24	1.46842E-10	1.39	0.16	0.09
2139	35.21	35.27	35.90	35.46	2.11581E-11	0.38	8.45454E-11	0.25	0.29	0.17
2140	35.63	35.75	35.04	35.47	2.09634E-11	0.38	8.67899E-11	0.24	0.31	0.18
2149	36.52	36.26	36.71	36.50	1.03136E-11	0.23	6.98102E-11	0.15	0.17	0.10
2185	35.62	35.10	35.79	35.50	2.0532E-11	0.36	1.36249E-10	0.15	0.28	0.16
2182	30.99	30.96	30.90	30.95	4.82083E-10	0.05	2.085E-09	0.23	0.16	0.09
2183	31.73	31.78	31.78	31.76	2.74337E-10	0.03	6.82517E-10	0.40	0.23	0.14
2093	36.72	36.74	36.12	36.53	1.01013E-11	0.35	7.49816E-11	0.13	0.28	0.16
2095	32.50	32.39	31.84	32.24	1.96693E-10	0.35	5.18192E-11	3.80	0.25	0.14
2096	34.08	34.08	33.99	34.05	5.62249E-11	0.05	1.27837E-10	0.44	0.17	0.10
2070	31.78	31.55	32.09	31.81	2.66219E-10	0.27	2.7002E-10	0.99	0.23	0.13
2074	36.59	36.41	36.08	36.36	1.13383E-11	0.26	1.03212E-10	0.11	0.23	0.13
2076	32.11	32.37	32.98	32.49	1.66165E-10	0.45	1.34451E-10	1.24	0.41	0.24
2215	32.50	32.10	32.71	32.44	1.72025E-10	0.31	2.04317E-10	0.84	0.20	0.12
2205	31.10	31.04	31.50	31.21	4.01652E-10	0.25	2.25504E-09	0.18	0.25	0.14
2204	33.57	33.09	33.33	33.33	9.26126E-11	0.24	9.19724E-10	0.10	0.27	0.16
2201	34.15	34.10	34.41	34.22	4.99751E-11	0.17	3.02869E-10	0.17	0.21	0.12
2200	32.00	32.17	32.59	32.25	1.95335E-10	0.30	8.8917E-10	0.22	0.25	0.14
2196	32.21	32.27	32.10	32.19	2.0363E-10	0.09	2.01377E-10	1.01	0.21	0.12
2195	31.08	31.02	31.40	31.17	4.14857E-10	0.20	1.79305E-09	0.23	0.36	0.21
2137	33.64	33.35	33.33	33.44	8.58138E-11	0.17	5.37388E-10	0.16	0.24	0.14
2105	35.02	35.09	34.99	35.03	2.84391E-11	0.05	2.6422E-10	0.11	0.16	0.10
2019	35.48	36.23	35.01	35.57	1.95596E-11	0.62	7.34811E-11	0.27	0.39	0.23

RBBP4 expression relative to HKs

	Efficiency Value:	**96**	**1.96**

Samples	CT 1	CT 2	CT 3	**Mean Ct**	**RBBP4 Transcript Relative Quantity**	**RRBP4 Standard Deviation**	**Mean HK Quantity**	**Ratio RRBP4/HK**	**Standard Deviation of Ratios**	**Standard Error**
2047	30.1	29.67	30.13	29.97	1.54496E-09	0.23	1.46842E-10	10.52	0.13	0.08
2139	31.05	30.28	30.61	30.65	1.03557E-09	0.20	8.45454E-11	12.25	0.12	0.07
2140	30.98	32.17	31.58	31.58	4.39716E-10	0.34	8.67899E-11	5.07	0.20	0.11
2149	32.17	31.58	32.27	32.01	3.90076E-10	0.35	6.98102E-11	5.59	0.20	0.12
2185	31.23	31.93	30.28	31.15	6.86338E-10	0.83	1.36249E-10	5.04	0.48	0.28
2182	26.26	26.53	26.85	26.55	1.42812E-08	0.18	2.085E-09	6.85	0.10	0.06
2183	29.02	29.40	28.89	29.10	2.64163E-09	0.26	6.82517E-10	3.87	0.15	0.09
2093	33.01	34.70	32.38	33.36	1.38339E-10	1.16	7.49816E-11	1.84	0.67	0.39
2095	31.36	31.87	32.53	31.92	3.51294E-10	0.37	5.18192E-11	6.78	0.21	0.12
2096	32.70	33.29	32.48	32.82	2.10151E-10	0.41	1.27837E-10	1.64	0.23	0.14
2070	29.58	29.48	30.00	29.69	1.76933E-09	0.26	2.7002E-10	6.55	0.15	0.09
2074	33.91	32.89	32.51	33.10	2.14469E-10	0.30	1.03212E-10	2.08	0.17	0.10
2076	32.22	31.96	32.20	32.13	3.53951E-10	0.12	1.34451E-10	2.63	0.07	0.04
2215	30.81	30.07	30.12	30.33	1.3021E-09	0.14	2.04317E-10	6.37	0.08	0.05
2205	28.67	28.57	28.57	28.60	3.83555E-09	0.02	2.25504E-09	1.70	0.01	0.01
2204	28.04	28.11	28.08	28.08	5.35514E-09	0.02	9.19724E-10	5.82	0.01	0.01
2201	31.25	31.04	30.95	31.08	7.32278E-10	0.07	3.02869E-10	2.42	0.04	0.02
2200	30.32	30.56	30.45	30.44	1.05526E-09	0.07	8.8917E-10	1.19	0.04	0.02
2196	32.30	32.27	32.52	32.36	2.90988E-10	0.13	2.01377E-10	1.44	0.07	0.04
2195	29.92	30.07	30.14	30.04	1.38404E-09	0.05	1.79305E-09	0.77	0.03	0.02
2137	30.47	30.90	30.73	30.70	8.65588E-10	0.11	5.37388E-10	1.61	0.06	0.04
2105	32.45	32.29	32.06	32.27	3.28512E-10	0.13	2.6422E-10	1.24	0.07	0.04
2019	32.74	32.33	33.49	32.85	2.06385E-10	0.58	7.34811E-11	2.81	0.34	0.19

TGFBI expression relative to HKs

	Efficiency Value:	**96**	**1.96**

Samples	CT 1	CT 2	CT 3	**Mean Ct**	**TGFBI Transcript Relative Quantity**	**TGFBI Standard Deviation**	**Mean HK Quantity**	**Ratio TGFBI/HK**	**Standard Deviation of Ratios**	**Standard Error**
2047	39.94	39.84	39.77	39.85	1.03035E-12	0.04	1.46842E-10	0.01	0.03	0.01
2139	36.34	36.29	36.33	36.32	1.17111E-11	0.02	8.45454E-11	0.14	0.01	0.01
2140	36.87	36.81	36.99	36.89	7.81622E-12	0.09	8.67899E-11	0.09	0.05	0.03
2149	36.26	36.29	36.23	36.26	1.21521E-11	0.03	6.98102E-11	0.17	0.02	0.01
2185	35.94	35.76	35.16	35.62	2.03902E-11	0.31	1.36249E-10	0.15	0.18	0.10
2182	32.95	32.94	32.87	32.92	1.23909E-10	0.04	2.085E-09	0.06	0.02	0.01
2183	35.36	35.05	35.36	35.26	2.49491E-11	0.16	6.82517E-10	0.04	0.09	0.05
2093	35.78	35.02	35.82	35.54	2.11581E-11	0.41	7.49816E-11	0.28	0.23	0.14
2095	38.42	38.16	38.66	38.41	2.73592E-12	0.25	5.18192E-11	0.05	0.14	0.08
2096	37.56	37.52	37.24	37.44	5.51414E-12	0.14	1.27837E-10	0.04	0.08	0.05
2070	35.39	35.55	35.39	35.44	2.11418E-11	0.08	2.7002E-10	0.08	0.05	0.03
2074	37.23	36.81	37.14	37.06	7.25917E-12	0.17	1.03212E-10	0.07	0.10	0.06
2076	35.43	35.69	36.00	35.71	1.67286E-11	0.17	1.34451E-10	0.12	0.10	0.06
2215	32.25	32.36	32.95	32.52	1.5255E-10	0.31	2.04317E-10	0.75	0.18	0.10
2205	31.42	31.02	31.25	31.23	4.14857E-10	0.13	2.25504E-09	0.18	0.07	0.04
2204	32.15	32.51	32.24	32.30	1.82675E-10	0.14	9.19724E-10	0.20	0.08	0.05
2201	32.29	32.21	32.55	32.35	1.8016E-10	0.17	3.02869E-10	0.59	0.10	0.06
2200	33.97	33.27	33.32	33.52	9.00801E-11	0.13	8.8917E-10	0.10	0.08	0.04
2196	34.67	34.70	34.21	34.53	4.17657E-11	0.25	2.01377E-10	0.21	0.14	0.08
2195	32.96	32.80	32.58	32.78	1.41351E-10	0.12	1.79305E-09	0.08	0.07	0.04
2137	32.06	32.56	32.66	32.43	1.59151E-10	0.12	5.37388E-10	0.30	0.07	0.04
2105	34.86	34.79	34.48	34.71	3.68384E-11	0.16	2.6422E-10	0.14	0.09	0.05
2019	33.03	33.94	33.25	33.41	8.04999E-11	0.36	7.34811E-11	1.10	0.21	0.12

Snap Frozen tissues

				HPRT	Efficiency Value:	**99.2**	**1.992**
	CT			**Mean Ct**	**HPRT Relative Quantity**	**Standard Deviation**	
Samples	1	2	3				
1985	27.36	27.15	27.25	27.25	2.86793E-09	0.11	
1978	27.15	27.57	26.87	27.20	2.98766E-09	0.35	
1973	31.02	31.57	32.05	31.55	1.29373E-10	0.52	
1974b	30.76	31.36	31.95	31.36	1.48388E-10	0.60	
1965c	29.26	29.06	29.18	29.17	7.20847E-10	0.10	
1964	28.03	28.55	28.49	28.36	1.2934E-09	0.28	
1966	32.13	32.14	32.20	32.16	8.32994E-11	0.04	
1965	28.60	28.95	28.86	28.80	9.36976E-10	0.18	
1967b	32.00	31.71	32.39	32.03	9.10542E-11	0.34	
2140	28.75	29.04	29.18	28.99	8.18877E-10	0.22	
2092	22.06	22.19	21.85	22.03	1.24095E-07	0.17	
2185	29.06	29.18	29.05	29.10	7.58201E-10	0.07	
2182	29.04	29.96	29.38	29.46	5.8331E-10	0.47	
2179	24.98	24.44	25.16	24.86	1.61342E-08	0.37	
2142	31.00	32.14	32.54	31.89	1.00735E-10	0.80	
2097	29.00	28.17	29.18	28.78	9.50599E-10	0.54	
2093	30.64	30.72	30.24	30.53	2.68824E-10	0.26	
2197	31.15	31.17	30.66	30.99	1.92878E-10	0.29	
2022	24.22	23.90	24.06	24.06	2.87412E-08	0.16	
2021	30.89	30.70	31.61	31.07	1.82935E-10	0.48	
2215	35.84	36.30	34.77	35.64	6.75852E-12	0.79	
2204	28.44	28.50	28.32	28.42	1.23561E-09	0.09	
2200	26.38	26.24	26.11	26.24	5.94496E-09	0.14	
2195	28.00	28.08	28.06	28.05	1.61772E-09	0.04	
2151	30.50	30.77	30.14	30.47	2.81397E-10	0.32	
2019	25.31	25.22	25.18	25.24	1.22937E-08	0.07	
2209	26.32	26.25	26.39	26.32	5.62494E-09	0.07	

2157	28.77	28.73	28.72	28.74	9.80799E-10	0.03
2144	25.37	26.90	27.06	26.44	5.14588E-09	0.93
2216	25.39	25.26	25.24	25.30	1.17727E-08	0.08

	TFRC	Efficiency Value:		**105.8**	**2.058**	
		CT		**Mean Ct**	**TFRC Relative Quantity**	**Standard Deviation**
Samples	1	2	3			
1985	33.91	33.92	33.81	33.88	6.32563E-11	0.06
1978	28.27	28.97	29.06	28.77	2.18963E-09	0.43
1973	33.15	33.57	33.05	33.26	9.74419E-11	0.28
1974b	29.74	29.85	30.10	29.90	1.00048E-09	0.18
1965c	31.26	31.05	31.28	31.20	4.06319E-10	0.13
1964	31.19	31.67	31.44	31.43	3.44845E-10	0.24
1966	33.13	33.47	33.21	33.27	9.65455E-11	0.18
1965	27.46	27.86	28.00	27.77	4.35907E-09	0.28
1967b	33.84	33.72	33.33	33.63	7.52248E-11	0.27
2140	27.03	27.11	27.28	27.14	6.76154E-09	0.13
2092	23.03	23.03	23.22	23.09	1.11741E-07	0.11
2185	28.04	28.18	27.97	28.06	3.56529E-09	0.11
2182	26.97	27.69	26.94	27.20	6.48611E-09	0.42
2179	28.21	28.14	28.33	28.23	3.18366E-09	0.10
2142	24.32	24.02	24.03	24.12	5.47209E-08	0.17
2097	29.55	29.69	29.99	29.74	1.11267E-09	0.22
2093	34.15	34.71	34.66	34.51	4.09693E-11	0.31
2197	30.24	30.03	30.01	30.09	8.72979E-10	0.13
2022	29.14	30.70	30.20	30.01	9.22755E-10	0.80
2021	30.38	30.38	30.26	30.34	7.35783E-10	0.07
2215	29.45	29.50	29.60	29.52	1.30196E-09	0.08
2204	28.36	28.02	28.19	28.19	3.26561E-09	0.17
2200	29.58	29.42	29.49	29.50	1.32014E-09	0.08
2195	31.79	31.78	31.51	31.69	2.87976E-10	0.16
2151	26.46	26.52	26.05	26.34	1.17454E-08	0.26
2019	24.97	25.33	25.01	25.10	2.77424E-08	0.20

2209	25.73	25.70	25.43	25.62	1.93915E-08	0.17
2157	27.96	27.70	27.52	27.73	4.50237E-09	0.22
2144	25.60	25.29	25.42	25.44	2.20191E-08	0.16

	PGBD	Efficiency Value:		**100.7**	**2**	
	CT			**Mean Ct**	**PGBD Relative Quantity**	**Standard Deviation**
Samples	1	2	3			
1985	34.71	34.43	34.81	34.65	1.07111E-11	0.20
1978	34.80	34.45	34.01	34.42	1.26664E-11	0.40
1973	36.11	37.67	36.05	36.61	2.56625E-12	0.92
1974b	36.41	37.05	37.05	36.84	2.17539E-12	0.37
1965c	35.09	35.48	35.18	35.25	6.91631E-12	0.20
1964	32.53	33.82	33.55	33.30	2.86578E-11	0.68
1966	35.75	37.08	36.20	36.34	3.11692E-12	0.68
1965	34.62	35.16	35.85	35.21	7.12096E-12	0.62
1967b	34.62	34.11	34.36	34.36	1.32006E-11	0.26
2140	28.75	28.53	28.25	28.51	9.41356E-10	0.25
2092	30.20	29.70	30.15	30.02	3.13866E-10	0.28
2185	29.01	29.12	29.07	29.07	6.27355E-10	0.06
2182	31.85	31.50	32.02	31.79	8.61605E-11	0.27
2179	30.55	31.40	30.77	30.91	1.64048E-10	0.44
2142	32.37	32.18	32.01	32.19	6.45244E-11	0.18
2097	29.07	29.13	29.07	29.09	6.16774E-10	0.03
2093	32.59	32.55	31.14	32.09	6.90674E-11	0.83
2197	34.00	34.66	34.59	34.42	1.26972E-11	0.36
2022	33.09	32.84	33.28	33.07	3.38892E-11	0.22
2021	35.07	34.88	34.76	34.90	8.9049E-12	0.16
2215	34.88	34.53	34.33	34.58	1.12719E-11	0.28
2204	32.43	33.28	32.84	32.85	3.97844E-11	0.43
2200	34.47	35.35	33.39	34.40	1.28212E-11	0.98
2195	34.80	34.15	33.37	34.11	1.59167E-11	0.72

2151	34.63	34.24	34.25	34.37	1.31047E-11	0.22
2019	33.39	33.16	33.07	33.21	3.06756E-11	0.17
2209	30.21	30.99	30.87	30.69	1.92118E-10	0.42
2157	33.78	34.06	33.22	33.69	2.16185E-11	0.43
2144	31.70	31.68	31.59	31.66	9.49558E-11	0.06
2216	31.20	31.06	31.15	31.14	1.38724E-10	0.07

AKAP1			Efficiency Value:	**82.7**	**1.827**					
	CT			**Mean Ct**	**AKAP1 Relative Transcript Quantity**	**AKAP1 Standard Deviation**	**Mean HK Quantity**	**Ratio AKAP1/HK**	**Standard Deviation of Ratios**	**Standard Error**
Samples	1	2	3							
1985	30.19	30.48	30.28	30.32	1.20055E-08	0.15	9.80633E-10	12.24	0.12	0.07
1978	32.35	32.77	32.91	32.68	2.90269E-09	0.29	1.72998E-09	1.68	0.28	0.16
1973	34.16	33.25	34.21	33.87	1.41304E-09	0.54	7.64604E-11	18.48	0.48	0.28
1974b	34.76	31.13	31.36	32.42	3.39413E-09	2.03	3.8368E-10	8.85	1.20	0.69
1965c	32.67	32.39	33.01	32.69	2.8795E-09	0.31	3.78028E-10	7.62	0.20	0.12
1964	32.28	33.50	31.44	32.41	3.41461E-09	1.04	5.55635E-10	6.15	0.65	0.38
1966	33.78	33.68	33.75	33.74	1.53413E-09	0.05	6.09873E-11	25.15	0.24	0.14
1965	28.50	28.66	29.45	28.87	2.86643E-08	0.51	1.76772E-09	16.22	0.38	0.22
1967b	33.30	34.33	34.35	33.99	1.31463E-09	0.60	5.98265E-11	21.97	0.39	0.22
2140	27.43	27.52	27.33	27.43	6.83015E-08	0.10	2.84059E-09	24.04	0.13	0.08
2092	29.60	29.38	30.28	29.75	1.68484E-08	0.47	7.87167E-08	0.21	0.29	0.17
2185	31.73	31.87	31.64	31.75	5.07896E-09	0.12	1.65028E-09	3.08	0.08	0.05
2182	32.67	31.31	37.37	33.78	1.49166E-09	3.18	2.38519E-09	0.63	1.85	1.07
2179	31.63	31.39	31.88	31.63	5.43732E-09	0.25	6.49398E-09	0.84	0.24	0.14
2142	28.16	28.23	28.13	28.17	4.35867E-08	0.05	1.82954E-08	2.38	0.28	0.16
2097	28.98	28.95	28.62	28.85	2.90112E-08	0.20	8.93346E-10	32.47	0.23	0.13
2093	32.62	32.25	32.14	32.34	3.56147E-09	0.25	1.26287E-10	28.20	0.34	0.20
2197	30.53	30.88	30.46	30.62	9.98298E-09	0.23	3.59518E-10	27.77	0.21	0.12
2022	31.17	31.06	29.85	30.69	9.57132E-09	0.73	9.89927E-09	0.97	0.51	0.29
2021	32.22	32.33	32.02	32.19	3.88999E-09	0.16	3.09208E-10	12.58	0.19	0.11
2215	32.10	32.19	32.04	32.11	4.08178E-09	0.08	4.39997E-10	9.28	0.28	0.16
2204	28.81	28.88	28.47	28.72	3.13711E-08	0.22	1.51367E-09	20.73	0.20	0.12

2200	30.09	29.89	30.06	30.01	1.44089E-08	0.11	2.42597E-09	5.94	0.34	0.19
2195	31.43	31.01	31.60	31.35	6.46071E-09	0.30	6.40536E-10	10.09	0.30	0.17
2151	30.17	30.82	30.91	30.63	9.9231E-09	0.40	4.01329E-09	2.47	0.28	0.16
2019	29.13	29.15	29.24	29.17	2.38831E-08	0.06	1.33556E-08	1.79	0.09	0.05
2209	26.18	25.89	26.19	26.09	1.52941E-07	0.17	8.40285E-09	18.20	0.18	0.10
2157	28.09	28.14	28.10	28.11	4.52794E-08	0.03	1.83493E-09	24.68	0.16	0.09
2144	25.54	25.45	25.41	25.47	2.22078E-07	0.07	9.08666E-09	24.44	0.32	0.18
2216	28.10	28.01	28.28	28.13	4.47379E-08	0.14	6.77754E-09	6.60	0.11	0.07

APRIL		Efficiency Value:	**96**	**1.96**					
	CT		**Mean Ct**	**APRIL Relative Transcript Quantity**	**APRIL Standard Deviation**	**Mean HK Quantity**	**Ratio APRIL/HK**	**Standard Deviation of Ratios**	**Standard Error**
Samples	1	2 3							
1985	34.41	31.59 32.08	32.69	2.78724E-10	1.51	9.80633E-10	0.28	0.87	0.50
1978	26.17	26.98 26.15	26.43	1.88234E-08	0.47	1.72998E-09	10.88	0.36	0.21
1973	30.27	29.93 30.08	30.09	1.6034E-09	0.17	7.64604E-11	20.97	0.38	0.22
1974b	28.63	28.38 28.48	28.50	4.69544E-09	0.13	3.8368E-10	12.24	0.25	0.15
1965c	28.12	28.48 28.62	28.41	4.98861E-09	0.26	3.78028E-10	13.20	0.17	0.10
1964	31.25	31.21 30.98	31.15	7.89219E-10	0.15	5.55635E-10	1.42	0.27	0.16
1966	31.66	31.97 31.59	31.74	5.2941E-10	0.20	6.09873E-11	8.68	0.26	0.15
1965	25.65	25.75 25.52	25.64	3.21036E-08	0.12	1.76772E-09	18.16	0.24	0.14
1967b	31.24	31.99 31.08	31.44	6.49297E-10	0.49	5.98265E-11	10.85	0.33	0.19
2140	25.98	25.09 25.57	25.55	3.41847E-08	0.45	2.84059E-09	12.03	0.28	0.16
2092	25.69	25.59 25.71	25.66	3.16035E-08	0.06	7.87167E-08	0.40	0.12	0.07
2185	28.61	28.18 28.33	28.37	5.10177E-09	0.22	1.65028E-09	3.09	0.13	0.08
2182	30.32	30.04 30.66	30.34	1.35816E-09	0.31	2.38519E-09	0.57	0.29	0.17
2179	27.02	26.90 27.07	27.00	1.28843E-08	0.09	6.49398E-09	1.98	0.20	0.12
2142	25.54	25.09 25.86	25.50	3.53545E-08	0.39	1.82954E-08	1.93	0.36	0.21
2097	26.82	26.77 26.47	26.69	1.5873E-08	0.19	8.93346E-10	17.77	0.22	0.13
2093	30.68	30.72 30.06	30.49	1.23051E-09	0.37	1.26287E-10	9.74	0.37	0.22
2197	32.73	33.19 33.24	33.05	2.18757E-10	0.28	3.59518E-10	0.61	0.23	0.13
2022	27.06	27.09 27.20	27.12	1.18847E-08	0.07	9.89927E-09	1.20	0.28	0.16
2021	30.29	29.89 29.86	30.01	1.69208E-09	0.24	3.09208E-10	5.47	0.22	0.13

2215	30.32	30.51	30.45	30.43	1.28121E-09	0.10	4.39997E-10	2.91	0.28	0.16
2204	28.01	28.14	38.30	31.48	6.29223E-10	5.90	1.51367E-09	0.42	3.41	1.97
2200	26.96	26.90	27.03	26.96	1.31766E-08	0.07	2.42597E-09	5.43	0.33	0.19
2195	28.35	28.30	28.36	28.34	5.22922E-09	0.03	6.40536E-10	8.16	0.25	0.14
2151	26.49	26.17	26.21	26.29	2.07295E-08	0.17	4.01329E-09	5.17	0.18	0.11
2019	24.53	24.41	24.94	24.63	6.34903E-08	0.28	1.33556E-08	4.75	0.18	0.11
2209	25.10	25.20	25.12	25.14	4.49451E-08	0.05	8.40285E-09	5.35	0.16	0.09
2157	26.02	25.92	25.98	25.97	2.56529E-08	0.05	1.83493E-09	13.98	0.16	0.09
2144	25.99	25.30	25.40	25.56	3.38034E-08	0.37	9.08666E-09	3.72	0.38	0.22
2216	24.30	24.62	24.34	24.42	7.29637E-08	0.17	6.77754E-09	10.77	0.13	0.08

CEBPα			Efficiency Value:	**98.4**	1.985					
	CT			**Mean Ct**	**C/EBPalpha Relative Transcript Quantity**	**C/EBPalpha Standard Deviation**	**Mean HK Quantity**	**Ratio C/EBPalpha/HK**	**Standard Deviation of Ratios**	**Standard Error**
Samples	1	2	3							
1985	29.30	29.01	29.35	29.22	1.99268E-09	0.18	9.80633E-10	2.03	0.13	0.08
1978	27.01	27.13	27.06	27.07	8.72199E-09	0.06	1.72998E-09	5.04	0.23	0.13
1973	31.76	31.74	31.30	31.60	3.89734E-10	0.26	7.64604E-11	5.10	0.39	0.23
1974b	29.44	29.65	29.31	29.47	1.68264E-09	0.17	3.8368E-10	4.39	0.26	0.15
1965c	31.87	31.22	32.09	31.73	3.57315E-10	0.45	3.78028E-10	0.95	0.28	0.16
1964	28.45	28.67	28.23	28.45	3.37842E-09	0.22	5.55635E-10	6.08	0.29	0.17
1966	31.11	31.54	31.05	31.23	5.01126E-10	0.27	6.09873E-11	8.22	0.28	0.16
1965	28.75	29.60	29.40	29.25	1.95211E-09	0.44	1.76772E-09	1.10	0.35	0.20
1967b	32.41	32.29	32.75	32.48	2.1269E-10	0.24	5.98265E-11	3.56	0.22	0.13
2140	32.17	32.52	32.63	32.44	2.19103E-10	0.24	2.84059E-09	0.08	0.18	0.11
2092	26.97	26.58	26.48	26.68	1.13957E-08	0.26	7.87167E-08	0.14	0.19	0.11
2185	29.50	30.02	29.87	29.80	1.34193E-09	0.27	1.65028E-09	0.81	0.16	0.09
2182	32.48	32.40	32.31	32.40	2.25711E-10	0.09	2.38519E-09	0.09	0.23	0.13
2179	28.41	28.21	28.09	28.24	3.91053E-09	0.16	6.49398E-09	0.60	0.22	0.13
2142	27.62	28.03	27.80	27.82	5.21549E-09	0.21	1.82954E-08	0.29	0.30	0.17
2097	29.30	29.68	29.56	29.51	1.62965E-09	0.19	8.93346E-10	1.82	0.22	0.13
2093	32.20	32.09	32.44	32.24	2.50731E-10	0.18	1.26287E-10	1.99	0.32	0.19
2197	30.29	30.73	30.88	30.63	7.56141E-10	0.31	3.59518E-10	2.10	0.24	0.14

2022	26.04	26.26	26.65	26.32	1.4586E-08	0.31	9.89927E-09	1.47	0.33	0.19
2021	34.62	34.73	34.78	34.71	4.62096E-11	0.08	3.09208E-10	0.15	0.18	0.10
2215	29.02	29.76	29.70	29.49	1.65215E-09	0.41	4.39997E-10	3.75	0.37	0.21
2204	32.67	31.95	32.16	32.26	2.47883E-10	0.37	1.51367E-09	0.16	0.26	0.15
2200	28.76	28.53	28.46	28.58	3.08327E-09	0.16	2.42597E-09	1.27	0.34	0.20
2195	35.07	35.51	34.95	35.18	3.35566E-11	0.29	6.40536E-10	0.05	0.30	0.17
2151	32.22	32.38	33.61	32.74	1.78778E-10	0.76	4.01329E-09	0.04	0.47	0.27
2019	31.01	30.72	30.35	30.69	7.25667E-10	0.33	1.33556E-08	0.05	0.21	0.12
2209	29.38	29.27	29.34	29.33	1.84792E-09	0.06	8.40285E-09	0.22	0.16	0.09
2157	29.04	29.26	28.65	28.98	2.34373E-09	0.31	1.83493E-09	1.28	0.24	0.14
2144	31.34	31.98	31.01	31.44	4.33927E-10	0.49	9.08666E-09	0.05	0.43	0.25
2216	27.04	27.26	27.65	27.32	7.34811E-09	0.31	6.77754E-09	1.08	0.20	0.11

DDB2		Efficiency Value:		**87.2**	**1.872**					
	CT			**Mean Ct**	**DDB2 Relative Transcript Quantity**	**DDB2 Standard Deviation**	**Mean HK Quantity**	**Ratio DDB2/HK**	**Standard Deviation of Ratios**	**Standard Error**
Samples	1	2	3							
1985	29.05	28.98	28.86	28.96	1.33832E-08	0.10	9.80633E-10	13.65	0.10	0.05
1978	27.56	27.13	27.08	27.26	3.89497E-08	0.26	1.72998E-09	22.51	0.27	0.16
1973	32.65	32.84	32.98	32.82	1.19468E-09	0.17	7.64604E-11	15.62	0.38	0.22
1974b	29.30	29.43	29.32	29.35	1.05063E-08	0.07	3.8368E-10	27.38	0.24	0.14
1965c	33.64	33.87	33.88	33.80	6.49621E-10	0.14	3.78028E-10	1.72	0.12	0.07
1964	28.12	28.18	28.61	28.30	2.02291E-08	0.27	5.55635E-10	36.41	0.30	0.17
1966	31.63	31.48	31.74	31.62	2.54258E-09	0.13	6.09873E-11	41.69	0.25	0.14
1965	29.36	29.54	29.82	29.57	9.13561E-09	0.23	1.76772E-09	5.17	0.27	0.16
1967b	32.67	32.54	32.61	32.61	1.36821E-09	0.07	5.98265E-11	22.87	0.17	0.10
2140	25.23	25.30	25.40	25.31	1.31731E-07	0.09	2.84059E-09	46.37	0.13	0.07
2092	26.31	29.25	29.22	28.26	2.07853E-08	1.69	7.87167E-08	0.26	0.98	0.57
2185	27.01	26.99	26.58	26.86	4.99269E-08	0.24	1.65028E-09	30.25	0.15	0.09
2182	31.88	32.40	32.08	32.12	1.85544E-09	0.26	2.38519E-09	0.78	0.27	0.16
2179	29.66	30.60	29.52	29.93	7.32296E-09	0.59	6.49398E-09	1.13	0.39	0.23
2142	29.52	29.53	29.36	29.47	9.74603E-09	0.10	1.82954E-08	0.53	0.28	0.16
2097	29.01	29.22	29.45	29.23	1.13495E-08	0.22	8.93346E-10	12.70	0.23	0.13

2093	31.85	31.87	31.71	31.81	2.25278E-09	0.09	1.26287E-10	17.84	0.31	0.18
2197	32.70	32.77	32.20	32.56	1.4117E-09	0.31	3.59518E-10	3.93	0.24	0.14
2022	31.48	31.10	31.04	31.21	3.28647E-09	0.24	9.89927E-09	0.33	0.31	0.18
2021	32.49	31.69	32.39	32.19	1.7759E-09	0.44	3.09208E-10	5.74	0.30	0.18
2215	33.79	34.15	32.58	33.51	7.78922E-10	0.82	4.39997E-10	1.77	0.55	0.32
2204	30.19	30.33	30.29	30.27	5.90683E-09	0.07	1.51367E-09	3.90	0.16	0.09
2200	32.07	32.88	32.05	32.33	1.62351E-09	0.47	2.42597E-09	0.67	0.43	0.25
2195	33.41	33.11	33.05	33.19	9.4968E-10	0.19	6.40536E-10	1.48	0.27	0.16
2151	33.25	33.33	33.31	33.30	8.88343E-10	0.04	4.01329E-09	0.22	0.16	0.09
2019	31.99	31.09	31.02	31.37	2.97327E-09	0.54	1.33556E-08	0.22	0.32	0.19
2209	29.06	28.74	29.02	28.94	1.35801E-08	0.17	8.40285E-09	1.62	0.18	0.11
2157	29.30	29.35	29.89	29.51	9.48523E-09	0.33	1.83493E-09	5.17	0.25	0.14
2144	31.21	31.33	30.40	30.98	3.78745E-09	0.51	9.08666E-09	0.42	0.43	0.25
2216	28.29	28.06	28.12	28.16	2.21741E-08	0.12	6.77754E-09	3.27	0.11	0.06

Granulin		Efficiency Value:		**109**	**2.018**					
	CT			**Mean Ct**	**Granulin Relative Transcript Quantity**	**Granulin Standard Deviation**	**Mean HK Quantity**	**Ratio Granulin/HK**	**Standard Deviation of Ratios**	**Standard Error**
Samples	1	2	3							
1985	27.07	27.88	27.89	27.61	3.80286E-09	0.47	9.80633E-10	3.88	0.28	0.16
1978	26.20	26.23	26.38	26.27	9.7661E-09	0.10	1.72998E-09	5.65	0.23	0.14
1973	33.30	33.20	32.91	33.14	7.86917E-11	0.20	7.64604E-11	1.03	0.38	0.22
1974b	32.02	31.19	31.27	31.49	2.49469E-10	0.46	3.8368E-10	0.65	0.36	0.21
1965c	34.05	33.88	36.28	34.74	2.55891E-11	1.34	3.78028E-10	0.07	0.78	0.45
1964	29.54	29.03	29.28	29.28	1.17731E-09	0.26	5.55635E-10	2.12	0.30	0.17
1966	30.31	30.15	30.24	30.23	6.0425E-10	0.08	6.09873E-11	9.91	0.24	0.14
1965	29.96	29.24	28.74	29.31	1.15277E-09	0.61	1.76772E-09	0.65	0.42	0.24
1967b	31.62	31.66	32.41	31.90	1.87945E-10	0.45	5.98265E-11	3.14	0.31	0.18
2140	25.17	25.18	25.02	25.12	2.18456E-08	0.09	2.84059E-09	7.69	0.13	0.07
2092	22.79	22.76	22.58	22.71	1.18916E-07	0.11	7.87167E-08	1.51	0.13	0.08
2185	25.69	25.86	25.84	25.80	1.3616E-08	0.09	1.65028E-09	8.25	0.07	0.04
2182	27.63	27.74	28.51	27.96	2.98129E-09	0.48	2.38519E-09	1.25	0.36	0.21
2179	25.36	24.97	25.12	25.15	2.14404E-08	0.20	6.49398E-09	3.30	0.23	0.13

2142	23.72	23.41	23.54	23.56	6.56257E-08	0.16	1.82954E-08	3.59	0.29	0.17
2097	25.97	25.32	25.57	25.62	1.54142E-08	0.33	8.93346E-10	17.25	0.27	0.16
2093	28.58	28.55	28.63	28.59	1.92008E-09	0.04	1.26287E-10	15.20	0.31	0.18
2197	36.23	32.50	33.68	34.14	3.89949E-11	1.91	3.59518E-10	0.11	1.11	0.64
2022	24.93	24.61	24.53	24.69	2.9614E-08	0.21	9.89927E-09	2.99	0.31	0.18
2021	27.28	27.35	27.47	27.37	4.52193E-09	0.10	3.09208E-10	14.62	0.18	0.10
2215	27.71	27.65	27.32	27.56	3.94796E-09	0.21	4.39997E-10	8.97	0.30	0.18
2204	26.17	26.18	26.07	26.14	1.06994E-08	0.06	1.51367E-09	7.07	0.16	0.09
2200	25.76	25.88	25.59	25.74	1.41356E-08	0.15	2.42597E-09	5.83	0.34	0.20
2195	28.72	28.52	28.75	28.66	1.81946E-09	0.13	6.40536E-10	2.84	0.26	0.15
2151	23.51	23.62	23.56	23.56	6.53192E-08	0.06	4.01329E-09	16.28	0.16	0.09
2019	22.30	22.45	22.33	22.36	1.52042E-07	0.08	1.33556E-08	11.38	0.10	0.06
2209	22.14	22.01	21.99	22.05	1.89454E-07	0.08	8.40285E-09	22.55	0.16	0.09
2157	25.46	25.47	25.58	25.50	1.67299E-08	0.07	1.83493E-09	9.12	0.17	0.10
2144	23.49	23.26	23.41	23.39	7.39453E-08	0.12	9.08666E-09	8.14	0.32	0.19
2216	24.07	24.33	24.05	24.15	4.32667E-08	0.16	6.77754E-09	6.38	0.12	0.07

NCOA3		Efficiency Value:		**104.8**	**2.048**					
	CT			**Mean Ct**	**NCOA3 Transcript Relative Quantity**	**NCOA3 Standard Deviation**	**Mean HK Quantity**	**Ratio NCOA3/HK**	**Standard Deviation of Ratios**	**Standard Error**
Samples	1	2	3							
1985	31.89	31.00	30.59	31.16	1.99048E-10	0.66	9.80633E-10	0.20	0.39	0.23
1978	28.19	28.35	27.88	28.14	1.7345E-09	0.24	1.72998E-09	1.00	0.27	0.15
1973	33.38	33.45	33.97	33.60	3.46189E-11	0.32	7.64604E-11	0.45	0.41	0.24
1974b	29.65	29.09	29.17	29.30	7.53346E-10	0.30	3.8368E-10	1.96	0.30	0.17
1965c	32.58	32.94	32.68	32.73	6.44366E-11	0.19	3.78028E-10	0.17	0.14	0.08
1964	31.04	31.21	31.73	31.33	1.76632E-10	0.36	5.55635E-10	0.32	0.33	0.19
1966	33.62	33.04	33.69	33.45	3.8549E-11	0.36	6.09873E-11	0.63	0.31	0.18
1965	30.22	30.80	30.93	30.65	2.86904E-10	0.38	1.76772E-09	0.16	0.32	0.18
1967b	35.03	35.53	32.93	34.50	1.82035E-11	1.38	5.98265E-11	0.30	0.81	0.47
2140	28.02	27.76	27.70	27.83	2.17133E-09	0.17	2.84059E-09	0.76	0.15	0.09
2092	32.67	31.95	32.16	32.26	9.04682E-11	0.37	7.87167E-08	0.00	0.24	0.14
2185	26.76	26.53	26.46	26.58	5.29434E-09	0.16	1.65028E-09	3.21	0.10	0.06

2182	30.07	30.51	30.95	30.51	3.17193E-10	0.44	2.38519E-09	0.13	0.34	0.20
2179	30.20	30.38	30.61	30.40	3.44039E-10	0.21	6.49398E-09	0.05	0.23	0.13
2142	28.01	28.72	28.35	28.36	1.48143E-09	0.36	1.82954E-08	0.08	0.35	0.20
2097	29.38	29.27	29.34	29.33	7.39082E-10	0.06	8.93346E-10	0.83	0.20	0.11
2093	34.71	33.84	33.63	34.06	2.48944E-11	0.57	1.26287E-10	0.20	0.45	0.26
2197	30.50	30.88	30.70	30.69	2.78129E-10	0.19	3.59518E-10	0.77	0.19	0.11
2022	28.40	28.26	28.53	28.40	1.443E-09	0.14	9.89927E-09	0.15	0.29	0.17
2021	31.62	31.73	31.78	31.71	1.34192E-10	0.08	3.09208E-10	0.43	0.18	0.10
2215	27.15	27.52	27.63	27.43	2.8786E-09	0.25	4.39997E-10	6.54	0.31	0.18
2204	26.97	26.58	26.48	26.68	4.9517E-09	0.26	1.51367E-09	3.27	0.22	0.12
2200	28.50	29.02	28.87	28.80	1.08327E-09	0.27	2.42597E-09	0.45	0.37	0.21
2195	27.82	27.41	27.32	27.52	2.71167E-09	0.27	6.40536E-10	4.23	0.29	0.17
2151	24.41	24.21	24.09	24.24	2.84708E-08	0.16	4.01329E-09	7.09	0.18	0.10
2019	24.61	24.03	24.80	24.48	2.39135E-08	0.40	1.33556E-08	1.79	0.25	0.14
2209	24.30	24.68	24.56	24.51	2.33488E-08	0.19	8.40285E-09	2.78	0.19	0.11
2157	26.20	26.09	26.09	26.13	7.3449E-09	0.06	1.83493E-09	4.00	0.16	0.10
2144	26.29	25.73	25.88	25.97	8.23757E-09	0.29	9.08666E-09	0.91	0.36	0.21
2216	24.04	24.02	24.65	24.24	2.84708E-08	0.36	6.77754E-09	4.20	0.22	0.13

RARRES3		Efficiency Value:		**101.1**	**2.01**					
	CT			**Mean Ct**	**RARRES3 Transcript Relative Quantity**	**RARRES3 Standard Deviation**	**Mean HK Quantity**	**Ratio RARRES3/HK**	**Standard Deviation of Ratios**	**Standard Error**
Samples	1	2	3							
1985	28.12	28.34	28.19	28.22	3.2058E-09	0.11	9.80633E-10	3.27	0.10	0.06
1978	27.37	27.28	27.19	27.28	6.13623E-09	0.09	1.72998E-09	3.55	0.23	0.14
1973	31.82	31.21	31.31	31.45	3.41673E-10	0.33	7.64604E-11	4.47	0.41	0.24
1974b	30.95	30.95	30.67	30.86	5.14301E-10	0.16	3.8368E-10	1.34	0.26	0.15
1965c	34.33	32.36	32.42	33.04	1.13494E-10	1.12	3.78028E-10	0.30	0.65	0.38
1964	28.45	28.17	28.46	28.36	2.90261E-09	0.16	5.55635E-10	5.22	0.28	0.16
1966	32.04	31.72	32.46	32.07	2.21291E-10	0.37	6.09873E-11	3.63	0.32	0.18
1965	29.37	28.74	28.78	28.96	1.91059E-09	0.35	1.76772E-09	1.08	0.31	0.18
1967b	31.49	30.56	31.10	31.05	4.49799E-10	0.47	5.98265E-11	7.52	0.32	0.18
2140	27.41	27.64	27.54	27.53	5.15993E-09	0.12	2.84059E-09	1.82	0.14	0.08

2092	26.40	26.20	26.40	26.33	1.18271E-08	0.12	7.87167E-08	0.15	0.13	0.08
2185	27.45	27.60	27.69	27.58	4.98417E-09	0.12	1.65028E-09	3.02	0.08	0.05
2182	27.16	27.20	27.95	27.44	5.50478E-09	0.45	2.38519E-09	2.31	0.34	0.20
2179	26.74	26.67	26.59	26.67	9.38714E-09	0.08	6.49398E-09	1.45	0.20	0.12
2142	26.52	26.01	26.59	26.37	1.15036E-08	0.32	1.82954E-08	0.63	0.33	0.19
2097	27.40	27.27	27.59	27.42	5.56875E-09	0.16	8.93346E-10	6.23	0.22	0.12
2093	29.04	29.80	29.82	29.55	1.26929E-09	0.44	1.26287E-10	10.05	0.40	0.23
2197	30.50	30.45	30.67	30.54	6.40537E-10	0.12	3.59518E-10	1.78	0.17	0.10
2022	29.87	29.74	29.18	29.60	1.23173E-09	0.37	9.89927E-09	0.12	0.35	0.20
2021	31.26	31.27	31.13	31.22	3.99801E-10	0.08	3.09208E-10	1.29	0.18	0.10
2215	26.49	27.79	27.43	27.24	6.32334E-09	0.67	4.39997E-10	14.37	0.48	0.28
2204	24.62	24.65	24.82	24.70	3.67758E-08	0.11	1.51367E-09	24.30	0.17	0.10
2200	24.15	24.66	24.55	24.45	4.35325E-08	0.27	2.42597E-09	17.94	0.37	0.21
2195	26.28	26.98	26.75	26.67	9.36548E-09	0.36	6.40536E-10	14.62	0.32	0.18
2151	24.25	24.33	24.41	24.33	4.74177E-08	0.08	4.01329E-09	11.82	0.16	0.09
2019	25.66	25.19	22.17	24.34	4.70901E-08	1.89	1.33556E-08	3.53	1.10	0.63
2209	23.16	23.19	23.33	23.23	1.01877E-07	0.09	8.40285E-09	12.12	0.16	0.09
2157	23.87	23.98	24.10	23.98	6.02972E-08	0.12	1.83493E-09	32.86	0.17	0.10
2144	26.99	26.07	26.14	26.40	1.1293E-08	0.51	9.08666E-09	1.24	0.43	0.25
2216	25.09	24.93	25.15	25.06	2.86544E-08	0.11	6.77754E-09	4.23	0.11	0.06

RBBP4		Efficiency Value:		**96**	**1.96**					
	CT			**Mean Ct**	**RBBP4 Relative Transcript Quantity**	**RBBP4 Standard Deviation**	**Mean HK Quantity**	**Ratio RBBP4/HK**	**Standard Deviation of Ratios**	**Standard Error**
Samples	1	2	3							
1985	26.27	26.98	27.42	26.89	1.20727E-08	0.58	9.80633E-10	12.31	0.34	0.20
1978	24.05	23.90	23.73	23.89	9.20917E-08	0.16	1.72998E-09	53.23	0.25	0.14
1973	31.20	31.25	31.03	31.16	6.67471E-10	0.12	7.64604E-11	8.73	0.37	0.21
1974b	28.67	28.99	28.54	28.73	3.45942E-09	0.23	3.8368E-10	9.02	0.28	0.16
1965c	26.87	26.05	26.01	26.31	1.78812E-08	0.48	3.78028E-10	47.30	0.29	0.17
1964	27.08	27.14	27.01	27.08	1.06374E-08	0.07	5.55635E-10	19.14	0.26	0.15
1966	29.25	29.38	29.25	29.29	2.36648E-09	0.08	6.09873E-11	38.80	0.24	0.14
1965	32.01	32.65	32.87	32.51	2.67241E-10	0.45	1.76772E-09	0.15	0.35	0.20
1967b	32.30	32.27	32.01	32.19	3.31246E-10	0.16	5.98265E-11	5.54	0.19	0.11

2140	27.22	27.28	27.14	27.21	9.696E-09	0.07	2.84059E-09	3.41	0.13	0.07
2092	28.99	29.45	29.34	29.26	2.42057E-09	0.24	7.87167E-08	0.03	0.18	0.10
2185	27.49	27.49	27.43	27.47	8.14728E-09	0.03	1.65028E-09	4.94	0.05	0.03
2182	27.34	27.50	27.00	27.28	9.26747E-09	0.26	2.38519E-09	3.89	0.27	0.16
2179	23.03	23.23	23.15	23.14	1.53827E-07	0.10	6.49398E-09	23.69	0.20	0.12
2142	29.41	29.32	29.31	29.35	2.28243E-09	0.06	1.82954E-08	0.12	0.28	0.16
2097	25.72	25.42	25.84	25.66	2.77969E-08	0.22	8.93346E-10	31.12	0.23	0.13
2093	30.18	30.35	30.42	30.32	1.1824E-09	0.12	1.26287E-10	9.36	0.31	0.18
2197	29.41	29.78	29.42	29.54	2.00655E-09	0.21	3.59518E-10	5.58	0.20	0.12
2022	25.89	25.98	26.12	26.00	2.21238E-08	0.12	9.89927E-09	2.23	0.29	0.17
2021	29.50	28.60	28.91	29.00	2.8807E-09	0.46	3.09208E-10	9.32	0.31	0.18
2215	27.08	27.70	27.75	27.51	7.92929E-09	0.37	4.39997E-10	18.02	0.35	0.20
2204	24.27	24.18	24.23	24.23	7.34624E-08	0.05	1.51367E-09	48.53	0.16	0.09
2200	25.15	25.40	25.38	25.31	3.52419E-08	0.14	2.42597E-09	14.53	0.34	0.20
2195	28.28	28.21	27.99	28.16	5.10306E-09	0.15	6.40536E-10	7.97	0.26	0.15
2151	34.13	24.08	24.13	27.45	8.2772E-09	5.79	4.01329E-09	2.06	3.35	1.93
2019	29.07	29.69	23.03	27.26	9.3728E-09	3.68	1.33556E-08	0.70	2.13	1.23
2209	24.04	24.05	23.78	23.96	8.82208E-08	0.15	8.40285E-09	10.50	0.18	0.10
2157	24.04	24.12	23.99	24.05	8.28109E-08	0.07	1.83493E-09	45.13	0.17	0.10
2144	25.25	25.69	24.78	25.24	3.69549E-08	0.46	9.08666E-09	4.07	0.41	0.24
2216	22.83	22.90	23.10	22.94	1.75373E-07	0.14	6.77754E-09	25.88	0.12	0.07

TGFBI		Efficiency Value:		**100.2**	**2**					
	CT			**Mean Ct**	**TGFBI Relative Transcript Quantity**	**TGFBI Standard Deviation**	**Mean HK Quantity**	**Ratio TGFBI/HK**	**Standard Deviation of Ratios**	**Standard Error**
Samples	1	2	3							
1985	27.33	27.78	27.50	27.54	5.13615E-09	0.23	9.80633E-10	5.24	0.15	0.09
1978	27.35	27.12	27.21	27.23	6.36732E-09	0.12	1.72998E-09	3.68	0.24	0.14
1973	30.49	30.16	30.20	30.28	7.65259E-10	0.18	7.64604E-11	10.01	0.38	0.22
1974b	27.99	28.12	28.11	28.07	3.54066E-09	0.07	3.8368E-10	9.23	0.25	0.14
1965c	28.10	28.30	28.28	28.23	3.18366E-09	0.11	3.78028E-10	8.42	0.11	0.06
1964	28.74	28.89	28.52	28.72	2.26685E-09	0.19	5.55635E-10	4.08	0.28	0.16
1966	32.55	32.65	32.91	32.70	1.42993E-10	0.19	6.09873E-11	2.34	0.26	0.15

1965	26.45	26.12	26.69	26.42	1.11375E-08	0.29	1.76772E-09	6.30	0.29	0.17
1967b	32.27	32.88	32.58	32.58	1.56116E-10	0.31	5.98265E-11	2.61	0.24	0.14
2140	25.71	25.59	25.61	25.64	1.91688E-08	0.06	2.84059E-09	6.75	0.12	0.07
2092	31.39	32.39	32.41	32.06	2.22831E-10	0.58	7.87167E-08	0.00	0.36	0.21
2185	27.76	27.03	27.76	27.52	5.20784E-09	0.42	1.65028E-09	3.16	0.25	0.14
2182	30.48	30.48	30.39	30.45	6.81768E-10	0.05	2.38519E-09	0.29	0.23	0.13
2179	29.85	29.23	29.82	29.63	1.20082E-09	0.35	6.49398E-09	0.18	0.28	0.16
2142	29.18	29.36	29.31	29.28	1.53052E-09	0.09	1.82954E-08	0.08	0.28	0.16
2097	28.52	28.30	28.51	28.44	2.7397E-09	0.12	8.93346E-10	3.07	0.21	0.12
2093	32.04	32.11	30.54	31.56	3.1513E-10	0.89	1.26287E-10	2.50	0.60	0.34
2197	34.46	35.11	33.43	34.33	4.61995E-11	0.85	3.59518E-10	0.13	0.51	0.30
2022	32.57	32.05	31.57	32.06	2.22831E-10	0.50	9.89927E-09	0.02	0.40	0.23
2021	35.06	35.21	33.05	34.44	4.29069E-11	1.21	3.09208E-10	0.14	0.72	0.41
2215	32.10	32.54	31.96	32.20	2.02691E-10	0.30	4.39997E-10	0.46	0.33	0.19
2204	29.24	29.01	29.29	29.18	1.64416E-09	0.15	1.51367E-09	1.09	0.18	0.10
2200	30.58	31.06	30.81	30.82	5.2876E-10	0.24	2.42597E-09	0.22	0.36	0.21
2195	31.37	31.26	30.97	31.20	4.05382E-10	0.21	6.40536E-10	0.63	0.27	0.16
2151	29.72	29.54	29.71	29.66	1.18155E-09	0.10	4.01329E-09	0.29	0.17	0.10
2019	27.45	27.57	27.12	27.38	5.72531E-09	0.23	1.33556E-08	0.43	0.16	0.09
2209	26.00	26.29	26.48	26.26	1.24726E-08	0.24	8.40285E-09	1.48	0.21	0.12
2157	28.41	28.11	28.17	28.23	3.17631E-09	0.16	1.83493E-09	1.73	0.19	0.11
2144	27.20	27.14	27.26	27.20	6.48611E-09	0.06	9.08666E-09	0.71	0.32	0.18
2216	26.82	25.88	26.75	26.48	1.06591E-08	0.52	6.77754E-09	1.57	0.31	0.18

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