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Molecular characterisation of the Her2-Top2A amplicon in breast cancer

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MOLECULAR CHARACTERISATION OF THE HER2-TOP2A AMPLICON IN BREAST CANCER

Olivia Herd

A dissertation submitted to the Faculty of Health Sciences, University of Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2009

DECLARATION

I, Olivia Herd, declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

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_____ day of_____ 2009

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

Conference Poster Presentations:

1. **Herd, O**., Willem, P. Simultaneous Amplification of the HER2/neu Gene and Chromosome 17 Centromere in Breast Carcinoma Specimens. *48th Annual Congress of the Federation of South African Societies of Pathology,* Cape Town, July 2008.

2. **Herd, O**., Willem, P. Simultaneous Amplification of the HER2/neu Gene and Chromosome 17 Centromere in Breast Carcinoma Specimens. *South African Society for Human Genetics conference*, Cape Town, April 2009 (Prize awarded for poster).

Publications:

1. **Herd, O**., Willem, P. Amplification of the CEP17 internal control in FISH HER2 testing. (In preparation).

ABSTRACT

The HER2 gene is amplified in 20-30% of breast cancers, a common cancer amongst South African women. HER2 amplification is associated with a poor prognosis and predicts response to treatments such as Herceptin. The gold standard for HER2 testing is Fluorescent *in situ* Hybridisation (FISH) with dual colour probes for the HER2 gene and chromosome 17 centromere (CEP17) internal control. According to international guidelines, a HER2/CEP17 ratio >2.2 is considered positive. The HER2 FISH test is complicated by the emergence of ambiguous cases with increased CEP17 signals that cannot be accounted for by chromosome 17 polysomy (> 6 copies of CEP17) and that may hide true HER2 gene amplification.

The aims of this study were to characterise the HER2 amplicon, in particular the copy number of genes in the vicinity of the HER2 gene, and to design an alternative control probe that could clarify the HER2 gene status in ambiguous cases. In addition, results on 1558 breast cancer specimens sent for routine testing were analysed to determine the trends of HER2 amplification amongst South African women.

The rate of HER2 gene amplification was significantly higher ($p < 0.05$) in African patients (52%) than in Caucasian patients (43%). In Caucasian women, the rate of HER2 amplification in the younger group (68%) was significantly higher ($p < 0.05$) than in the general Caucasian group (43%), while the same was not seen in the African cohort.

Nineteen ambiguous cases with more than 9 copies of CEP17 were further investigated. FISH assays with four different probe kits (PathVysion HER-2: Poseidon Repeat free TOP2A, HER2, CEP17: and Vysis PML-RARA respectively) were performed to determine the copy number of the HER2, TOP2A, RARA genes and CEP17. An in-house dual colour probe kit was designed using the ACTG1 gene as a control for HER2. Of the 19 ambiguous cases, 16 had centromeric amplification, showing that CEP17 is no longer an adequate internal control in FISH HER2 testing. The TOP2A gene was only amplified in HER2 positive cases and the RARA gene was only amplified when the TOP2A gene was also amplified. FISH with ACTG1 as

a control clearly revealed HER2 amplification in ambiguous cases on image analysis and gave HER2/ACTG1 ratios significantly higher than HER2/CEP17 ratios. However, screening of an additional 40 unambiguous cases showed an increased copy number, although limited (≤ 8), of the ACTG1 gene in four patients; this warrants further testing to assess the value of this gene as a control. Interestingly, a trend was observed for ACTG1 increased copy number in HER2 negative cases, this may point to the presence of a driver gene whose amplification tends to be mutually exclusive from HER2 amplification.

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ABBREVIATIONS

1.	HER2 :	Human Epidermal Growth Factor II gene
2.	ErbB2:	Human Epidermal Growth Factor Protein receptor
3.	TKR:	Tyrosine Kinase Receptor
4.	DCIS:	Ductal Carcinoma in situ
5.	IDC/IBC	Invasive Ductal Carcinoma/ Invasive Breast Cancer
6.	ASCO/CAP	American Society of Clinical Oncology/College of American Pathologists
7.	ER	Estrogen (Oestrogen) Receptor
8.	PR	Progesterone Receptor
9.	CMF	Cyclophosphamide, Methotrxate, 5-Flourouracil
10.	TOP2A	Topoisomerase II alpha
11.	FDA	Food and Drug Association
12.	IHC	Immunohistchemistry
13.	FISH	Fluorescent *in situ* Hybridisation
14.	CEP17	Chromosome 17 centromere
15.	BAC	Bacterial Artificial Chromosome
16.	PCR	Polymerase Chain Reaction
17.	HSR	Homogeneously Staining Regions
18.	BFB	Breakage-Fusion-Bridge
19.	RARA	Retinoic Acid Receptor Alpha
20.	GRB7	Growth factor receptor-bound protein 7
21.	TRAF4- TNF	Receptor-associated factor 4
22.	PPARBF	Peroxisome proliferator activated receptor binding protein
23.	CDC6	Cell division cycle 6 homolog
24.	PCGF2	Polycomb group ring finger 2
25.	SYNGR2	Synaptogyrin 2
26.	PGS1	Phosphatidylglycerophosphate synthase 1
27.	PSCD1	Pleckstrin homology, sec 7
28.	BIRC5	Baculoviral IAP repeat-containing protein 5
29.	THRA1	Thyroid alpha receptor 1
30.	CCR7	Chemokine (C-C motif) recptor 7

1. INTRODUCTION

1.1. Breast cancer in South Africa

Breast cancer is one of the most common cancers amongst South African women, as documented by the National Cancer Registry's most recent report, published in 2004 (Mqoqi et al., 2004; Parkin et al., 2008). In this country, the lifetime risk for developing the disease is 1/13 in the white population of European ancestry (similar to rates in western countries), 1/63 in women of mixed ethnicity and 1/81 in black South African women (Kruger and Apffelstaedt, 2007). The situation is likely to worsen, as research shows that the incidence of breast cancer is rising rapidly in developing countries (Parkin et al., 2008). This is most likely due to a number of lifestyle changes that all heighten the risk of developing the disease. For example, improved socioeconomic circumstances lead to unfavourable dietary changes and a reduction in exercise, resulting in increased obesity. Increased affluence also results in delayed childbearing, lower parity and reduced breast feeding. Increased life expectancy is an additional factor, as more women are able to reach an age where there is an increased risk of developing breast cancer (Sitas et al., 2008; Parkin et al., 2008; Porter, 2008).

While prevention initiatives are essential, it is important to ensure that all women already affected by the disease are accurately diagnosed and treated. The treatment of breast cancer can be multifaceted (Benson et al., 2009). The first line of defence is surgery (mastectomy or lumpectomy) and/or radiation, followed by adjuvant therapies such as chemotherapy, hormonal therapy and targeted therapies, such as Herceptin (Trastuzumab) (Benson et al., 2009).

The staging and histological features of tumours play a major role in determining which of the above mentioned treatments are optimal (Benson et al., 2009; Esteva and Hortobagyi, 2004). Traditional prognostic factors include: lymph node status, tumour size, nuclear grade and histological grade (Esteva and Hortobagyi, 2004). In addition to these, there are very useful molecular prognostic markers such as: Estrogen receptors; Progesterone receptors; and the HER2 receptor gene status (Esteva and Hortobagyi, 2004).

1.2. The role of HER2 in the development of breast cancer

The HER2 gene, coding for the Epidermal Growth Factor Receptor (ErbB2), is a proto-oncogene located on the long arm of chromosome 17, 17q. This gene is amplified in 20-30% of invasive breast cancers (Slamon et al., 1987). HER2 DNA amplification results in the over-expression of the ErbB2 protein which results in increased breast cell proliferation, survival and motility, all of which can lead to the formation of a malignant breast tumour (Badache and Goncalves 2006; Ross et al., 2009). The ErbB2 protein belongs to the ErbB family of proteins. Before exploring the role of the ErbB2 protein in carcinogenesis, its expression in normal cells, its structure, as well as its interaction with ligands will be reviewed.

1.2.1. ErbB2 expression in normal breast cells

The ErbB protein family includes: the ErbB1, ErbB2, ErbB3 and ErbB4 epidermal growth factor receptors. These receptors are expressed in a variety of tissues and play a role in cell development, proliferation and differentiation (Yarden, 2001). They are significantly involved in breast development, which mostly occurs postnataly (Stern, 2003). All receptors have designated functions: the ErbB2 and ErbB4 play a role in lobuloalveolar differentiation and lactation; ErbB1 plays a role in promoting ductal growth and ErbB3 is expressed throughout development but it's exact role has not yet been fully elucidated (Olayioye et al., 2000).

1.2.2. The structure of the ErbB2 protein

The ErbB receptors are all tyrosine kinase receptors (TKR) (Hynes and Stern, 1994). TKRs play a role in transducing extracellular signals to the interior of cells in response to the surrounding environment (Olayioye et al., 2000). All receptors in the ErbB family are transmembrane proteins that contain: an extracellular peptide binding region (consisting of domains I, II, III and IV); a transmembrane helix domain; and an intracellular tyrosine kinase domain with a c-terminal tail (Burgess et al., 2003).

1.2.3. ErbB2 interaction with ligands and the other ErbB receptors

For the ErbB family of receptors to be activated, they are required to form dimers, either with an identical receptor (homodimer) or with a different receptor of the same family (heterodimer). Dimerization occurs in the following way (Burgess et al., 2003): in the absence of a ligand, domains II and IV of the extracellular domains interact with each other, resulting in a tethered state (**Figure 1**). The binding of a ligand to domains I and III causes the receptor to change from a tethered to extended conformation, with domains II and IV becoming exposed. The dimerisation arm of domain II is then able to interact with the domain II of another receptor, (**Figure 1**).

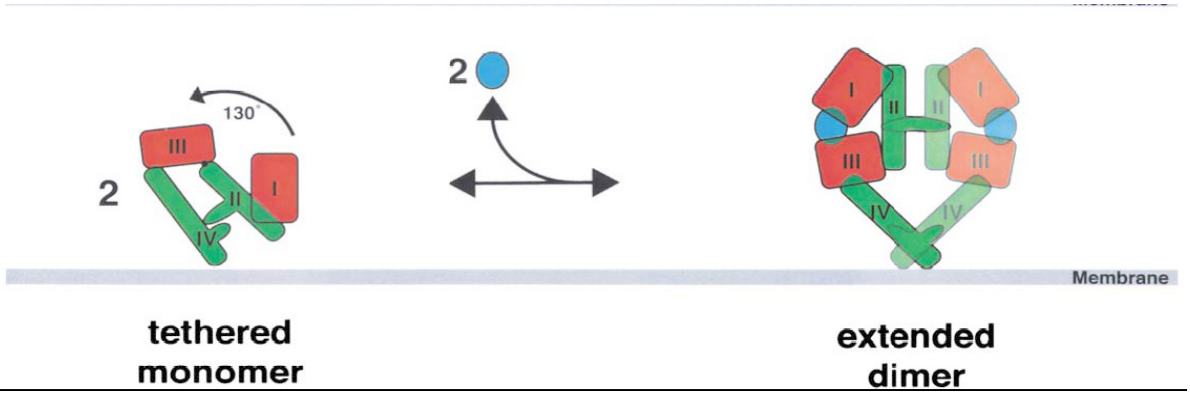

Figure 1: This diagram shows how binding of a ligand (blue circle) to domains I and III (in red), causes the extracellular domain to extend so that it is able to dimerize with an adjacent receptor, from Burgess et al., 2003.

The dimerisation of ErbB receptors leads to phosphorylation of the tyrosine residues on the intra-cellular c-terminal tail, allowing it to become a docking site for adaptor molecules. The recruitment of adaptor molecules is the primary event of the signalling cascade, which will instruct the cell to proliferate, survive or migrate (Burgess et al., 2003). The identity of the ligand and dimer combination determines which adaptor molecules are recruited and hence which signalling pathways are initiated (Yarden and Sliwkowski, 2001). **Figure 2** is a summary of all ErbB receptor dimer combinations, their relevant ligands, and their resultant signalling pathways.

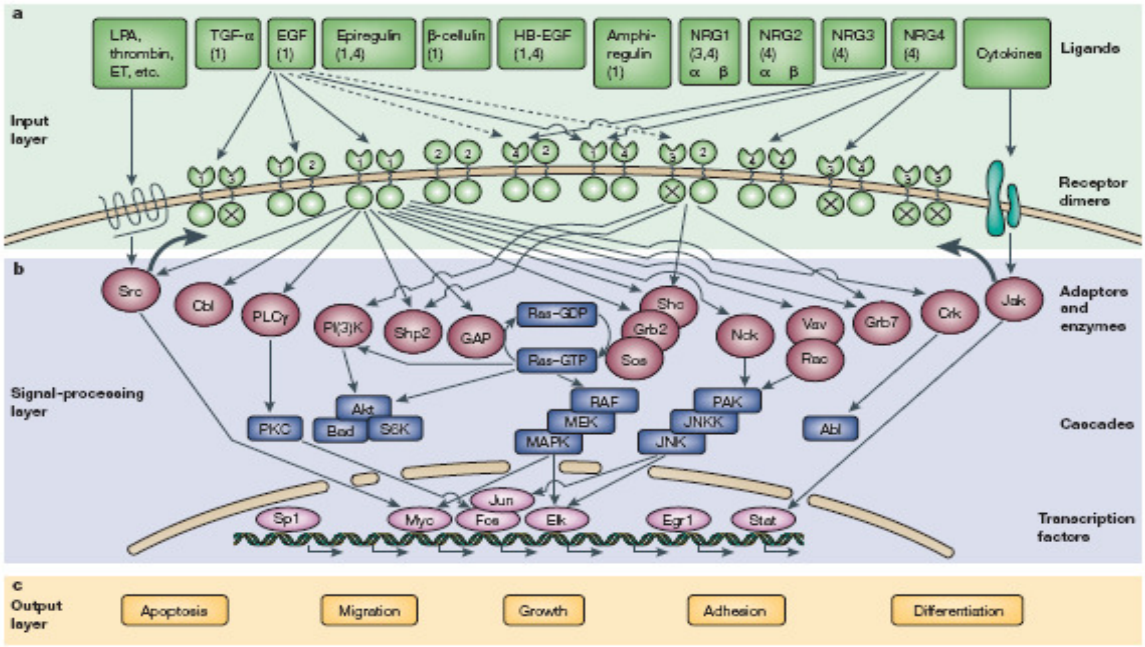

Figure 2: Summary of all signalling pathways induced by ErbB receptor dimmers, from Yarden and Sliwkowski, 2001.

The ErbB2 receptor is unique compared to the other receptors in the ErbB family. It differs in the following aspects:

1) Its extracellular domain permanently adopts an extended state and is therefore constantly poised for interaction with other receptors. This makes it the preferred heterodimerisation partner (Burgess et al., 2003) (**Figure 3**).
2) It has no known ligand and acts solely as a partner to which other receptors can combine (Rubin and Yarden, 2001) (**Figure 4**).
3) Heterodimers that include ErbB2 cause more potent signalling. When ErbB2 is present, there is increased ligand affinity and decreased internalization of the heterodimer-ligand complex (Baselga and Albanell, 2001).

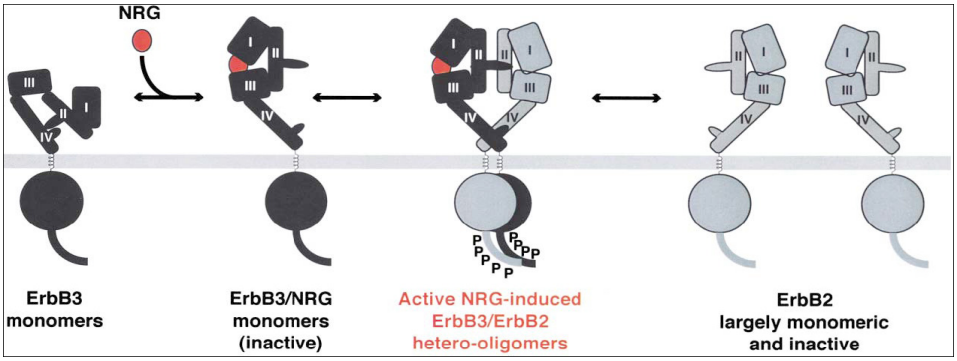

Figure 3: The ErbB2 receptor dimerising with ErbB3 receptor. The ErbB3 receptor requires a ligand to become untethered, while ErbB2 is always in an extended state, from Burgess et al., 2003. ErbB2 being in a constant tethered state results in it being a preferred heterodimerisation partner. Its overexpression thus leads to increased signalling and growth, resulting in carcinogenesis (Burgess et al., 2003)

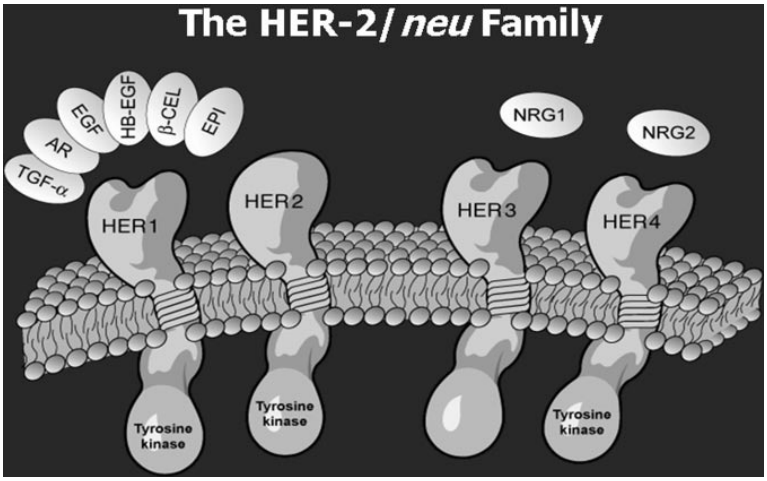

Figure 4: The ErbB family of receptors and their respective ligands. The ErbB2 receptor has no known ligands and the ErbB3 receptor has no intrinsic tyrosine kinase activity, from Ross et al., 2004. Due to it having no intrinsic tyrosine kinase activity, ErbB3 is required to bind to ErbB2 in order to become active. When there is an increase in ErBb2 receptors, there will be more opportunity for the activity of ErbB3.

1.2.4. HER2 in carcinogenesis

The HER2 gene amplification seen in breast cancer is paralleled by protein over expression and results in the increased dimerisation of ErbB receptors that become activated (Yarden and Sliwkowski, 2001). The resulting tyrosine kinase activity leads to the following biological effects in breast cells:

Increased cell proliferation:

This is due to the disruption of the cell cycle checkpoints, especially those involved in the transition of G1 to S. These are under the control of the RAS/Erk, p38MAPK, and P13K pathways, all of which are ErbB2 dependant (Badache and Goncalves, 2006).

Increased cell survival:

The Akt/P13K pathway, an important cell survival pathway, is activated by ErbB receptors. Over expression of ErbB2 receptor protein can over-stimulate this pathway and result in reduced apoptosis (Badache and Goncalves, 2006).

Increased cell motility:

Cell motility is a complex multi-step process and the ErbB signalling pathway influences many steps of this process. The ErbB2 receptor has indeed been shown to regulate breast cancer cell motility *in vitro* (Spencer et al., 2000, as cited in Badache and Goncalves, 2006), as well as motility in mice models (Guy et al., 1992, as cited in Badache and Goncalves, 2006)

1.3. Mechanism of HER2 amplification and the HER2 amplicon

Solid tumour progression is facilitated by the aberrant expression of genes that play a role in cell proliferation, apoptosis, genome stability, angiogenesis, invasion and metastasis (Albertson et al., 2003). Aberrant gene expression can be caused by many factors including: point mutations, epigenetic modifications or copy number alterations (amplification or deletion) (Albertson et al., 2003). For the ErbB2 protein, over expression can be caused by point mutations or truncation of the protein, however, gene amplification is by far the most common cause of its over expression (Ross et al., 2009).

The frequency of copy number changes amongst patients, as well as the generation of amplifications resulting in drug resistance, suggest that these events are due to selection that takes place during tumour progression (Albertson et al., 2003). These aberrations often point to genes, known as driver genes, that contribute to cancer formation or progression, and their study allows for the identification of potential target genes for therapy (Albertson, 2003; Myllykangas and Knuutila, 2006; Kitada and Yamasaki, 2008). Regions of amplification can present as double minutes (small chromatin bodies separated from the chromosomes), intrachromosomal contiguous segments, termed homogeneously staining regions (HSR), or distributed insertions interspersed amongst the genome (Albertson, 2003; Myllykangas and Knuutila, 2006). HER2 gene amplification appears to be in the form of HSRs (Guan et al., 1994; Muleris et al., 1995)

HSRs are formed by Breakage-Fusion-Bridge (BFB) cycles, a model of gene amplification proposed by McClintock in 1941 (McClintock, 1941). According to this model, the initiating event in the formation of HSRs is the uncapping of DNA sequence by a double stranded chromosomal break, or by a dysfunctional telomere (breakage). After DNA replication, the two uncapped sister chromatids fuse (fusion). Thereafter, during mitotic division, the dicentric fusion chromosome forms a bridge during anaphase segregation as chromosome centromeres are drawn to the opposite poles of the mitotic spindle (bridge) for cell division. Under this pull, a second DNA double-stranded break occurs and results in an inverted duplication of the DNA segment between the two consequential breakpoints. The BFB cycle proceeds with every cell division and eventually results in the increased copy number of a portion of a chromosome. The cells that contain an increased copy number of a gene that confers a proliferative cell growth advantage are selected for. An example of the BRB cycle, showing amplification of the gene denoted A is shown below in **Figure 5.** (Myllykangas and Knuutila, 2006; Kitada and Yamasaki, 2008; McClintock, 1941).

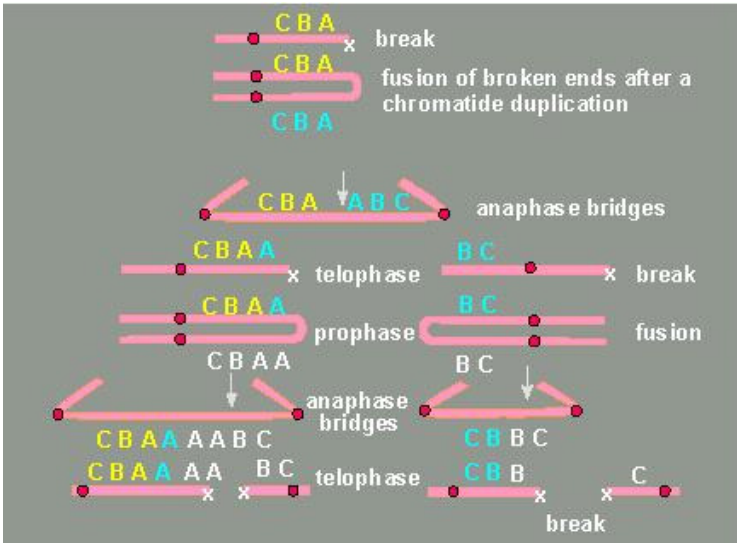

Figure 5: The Breakage-Fusion-Bridge cycle proposed by McClintock in 1941 that explains the formation of HSRs. (Image obtained from Botany online- Internet Hypertextbook, http://www.biologie.uni-hamburg.de/b-online/e21/21f.htm)

The HER2 gene is amplified in the form of HSRs due to BFB mechanisms, (Guan et al., 1994; Muleris et al., 1995). While HER2 is clearly the driver gene in this amplicon, it is also closely located to other genes such as: the THRA1, RARA, TOP2A, GRB7, TRAF4, PPARBP, CCR7, CDC6, and PCGF2 genes. It has been shown that these genes can all be co-amplified with the HER2 amplicon and that they may also play a role in both breast cancer tumorigenesis, and response to treatment (Kauraniemi et al., 2001; Jarvinen et al, 2006).

Of particular interest in the HER2 amplicon is the Topoisomerase IIα (TOP2A) gene, which is located very close to the HER2 gene, at 17q21.2, with only a 590 kilobase difference between the two genes (Hicks et al., 2005). The coamplification of the TOP2A gene along with the HER2 gene (probably as a result of the close proximity of these two genes) is now believed to play a role in the response of HER2 positive patients to anthracyclines (discussed below in section 1.4). Presently, the exact relationship between the amplification of these two genes remains unclear. It was initially believed that TOP2A alterations were restricted to HER2 amplified specimens; however some studies have now shown TOP2A copy number aberrations in patients with a normal HER2 status (Nielsen et al., 2008; Hicks et al., 2005). In addition to this, it was also originally believed that TOP2A and HER2 were co-

amplified in the same amplicon (Jarvinen et al., 1999), which is plausible due to the close proximity of the two genes. However, studies are beginning to suggest that they occur in separate amplicons. This is based on differing HER2 and TOP2A copy numbers in cases where both genes are amplified (Jarvinen et al., 1999; Olsen et al., 2004; Hicks et al., 2005; Bhargava et al., 2005). Additionally, a study done by Jarvinen et al. (1999) in which they performed fiber FISH, showed HER2 and TOP2A genes signals in separate DNA fibers, suggesting two distinct amplicons (Jarvinen et al., 1999).

Another gene that has previously been described in the HER2 amplicon is the retinoic acid receptor alpha, RARA, which plays a major role in Acute Promyelocytic Leukaemia (APL) (Kauraniemi et al., 2001). All-trans retinoic acid (ATRA), the therapy commonly used in APL patients, inhibits cell growth by binding to the retinoic acid receptors (RARs). This drug has been shown to have inhibitory effects on some human breast cancer cell lines (Tari et al., 2002). Studies have also shown that cells resistant to ATRA had high levels of ErbB2 expression (Rishi et al., 1996; Tari et al., 2002) and a study showed that ATRA-sensitive cells transfected with the HER2 gene developed resistance (Tari et al., 2002). In the same study by Tari et al. (2002), they also showed that cell lines resistant to ATRA had low levels of the RARA protein (Tari et al., 2002). The RARA gene is located in close proximity to the TOP2A gene and has been shown to be co-amplified with HER2, as well as with TOP2A in the same amplicon (Arriola et al., 2008; Keith et al., 1993). This poses an interesting therapeutic challenge when the RARA gene is co-amplified with the HER2 gene, since RARA amplification would result in ATRA sensitivity while HER2 amplification would result in ATRA resistance. The role of ATRA in the treatment of breast cancer is still experimental and clinical trials would still be needed to assess its efficacy as a clinical treatment.

1.4. The role of HER2 in breast cancer and its clinical significance

The HER2 gene status or its protein expression are very informative markers in breast cancer patients as they have both a predictive and prognostic value (Henry and Hayes, 2006).

1.4.1. Prognostic Value of HER2

The prognostic value of a marker is the ability of that marker to indicate the metastatic potential as well as the aggressiveness of a tumour (Henry and Hayes, 2006). HER2 has a high prognostic value, since the amplification of this gene is known to correlate with increased cell proliferation, cell motility, tumour invasiveness, progressive regional and distant metastases, accelerated angiogenesis, and reduced apoptosis- all of which result in a poorer clinical outcome (Rilke et al.,1991; Ross et al., 2009). The correlation was first illustrated in 1987 in a study by Slamon et al. (1987). They showed that HER2 amplification was able to independently predict the overall survival and the disease-free survival in a multivariate study of breast cancer patients (Slamon et al., 1987). This correlation between HER2 amplification and poor clinical outcome in **node-positive** patients has been proven to be true by many subsequent studies (Dowsett et al., 2000; Hynes and Stern, 1994).

In node negative patients, many papers initially yielded conflicting results. Some reviews claimed that HER2 amplification did not have any value in predicting which **node-negative** patients will relapse subsequent to surgery (Hynes and Stern, 1994; Dowsett et al., 2000; Rampaul et al., 2002; Menard et al., 2001; Elzagheid et al., 2006). However, numerous studies, all with large patient cohorts, showed that HER2 amplification is in fact an independent predictor of clinical outcome in node-negative patients (Paterson et al., 1991; Press et al., 1993; Press et al., 1997; Andulis et al., 1998; Harbeck et al., 1998; Tsutsui et al., 2002). It is now widely accepted that HER2 positivity is associated with a poorer prognosis in both node-negative and node-positive breast cancers, a view which has also been adopted by the American Society of Clinical Oncology and College of American Pathologists (ASCO/CAP) guidelines (Ross et al., 2009; Wolff et al., 2007).

1.4.2. Predictive value of HER2

If a molecular marker has predictive value, it can determine the sensitivity or resistance of a cancer to certain types of therapies (Henry and Hayes, 2006).
HER2 has predictive value for therapies such as hormonal therapy, chemotherapy and targeted therapies (Ross et al., 2009).

1.4.2.1. HER2 and hormonal therapy

Oestrogen and progesterone hormones can be instrumental to the growth of breast tumours (Utsumi et al., 2007). Hormonal therapies aim to stop these hormones from reaching the tumour cells, resulting in cessation of growth. The two main hormonal therapies are: Tamoxifen (blocks estrogen from entering into the cells) which is given to pre-menopausal women, and Aromatase Inhibitors (lowers the levels of oestrogen in the blood), given to post-menopausal women (Utsumi et al., 2007).

A range of studies have shown that HER2 amplification results in resistance to hormonal therapy (Dowsett et al., 2000; Rampaul et al., 2002; Hynes and Stern, 1994; Colomer et al., 2007). In 2003, the 20 year update of the Naples Gun Trial reached the same conclusion (De Placido et al., 2003). Two interesting studies showed that transfection of normal breast cancer cells with the HER2 gene resulted in oestrogen-independent growth and insensitivity to Tamoxifen (Benz et al., 1992; Pietras et al., 1995). One mechanism that may explain this resistance is that tumours that over express HER2 are likely to have reduced ER and PR receptor expression (Konecny et al., 2003). However, even if HER2 amplified tumours still have high levels of oestrogen receptors, the ErbB2 over-expression still causes the antiproliferative effects of hormone therapy to be impeded (Dowsett et al., 2001).

1.4.2.2. HER2 and chemotherapy

Other modes of treatment for breast cancer are represented by cytotoxic drugs. There are a number of cytotoxic agents and the most commonly used include: Anthracyclines (Doxorubicin, Epirubicin), Taxanes (Paclitaxel, Docetaxel),

Alkalylating agents (Cyclophosphamide), Fluoropyrimidines (5-FU) and Antimetabolites (Methotrexate) (Coleman, 2003). The above mentioned chemicals can be administered as single agents, but are usually given in combinations, which are more effective (Coleman, 2003).

Chemotherapy is a systemic treatment which can result in unpleasant side effects. Therefore, it is important that patients are given cytotoxic agents that are minimally harmful, and only when necessary. There are many combinations in which these agents can be administered, and it is helpful if doctors have clues as to which agents should be included or excluded into a treatment. HER2 amplification can be used in predicting the benefit from the following agents and/or combinations.

1.4.2.2.1. HER2 and CMF

One of the more popular chemotherapy combinations used in breast cancer is Cyclophosphamide, Methotrexate, 5-Flourouracil (CMF) (Thomas and Hortobagyi, 2003). Numerous studies have shown that HER2 amplification is associated with resistance to this combination (Berns et al., 1995; Stal et al., 1995; Giai et al., 1994; Gusterson et al., 1992; Allred et al., 1992). However, others studies have not seen this association (Miles et al., 1999; Menard et al., 2001). The issue of whether HER2 amplification results in CMF resistance remains contentious, however it is now widely accepted that anthracycline-containing regimes (instead of Methotrexate) give superior treatment results (Harris et al., 2007). According to the 2007 ASCO/CAP recommendations on tumour markers, it is recommended that anthracycline regimes be prescribed to all patients with HER2 amplification-making the issue of CMF resistance in amplified patients redundant (Harris et al., 2007).

1.4.2.2.2. HER2 and Taxanes

Other chemotherapeutic agents are represented by the Taxanes (Paclitaxel, Docetaxel), which target the formation of microtubules during cell division and results in cell apoptosis (Pusztai, 2007). In breast cancer, the Taxanes are widely used and have

shown significant success in the adjuvant setting; however, resistance appears to be a problem (Pusztai, 2007). HER2 amplification is a very useful marker when utilising Taxanes and Paclitaxel in particular. Patients that were Estrogen receptor (ER) and HER2 positive derived significant benefit from Paclitaxel in comparison to those that were Estrogen Receptor positive and HER2 negative (Pusztai, 2007; Andre, 2008).

1.4.2.2.3. HER2 and Anthracyclines

There is now overwhelming evidence that patients with HER2 amplification obtain the best benefit from chemotherapeutic combinations that contain anthracyclines and this is now the recommended treatment option, should chemotherapy be required (Muss et al., 1994; Paik et al., 1998; Thor et al., 1998; Paik et al., 2000; Piccart et al., 2001; Di Leo et al., 2002; Penault-Llorca et al., 2003; Tanner et al., 2006; Dhesy-Thind et al., 2007; Ross et al., 2009).

However, anthracyclines are believed to have little effect on ErbB2 and this has led to a heavily debated issue as to whether the increased sensitivity to anthracyclines in HER2 amplified cases is actually due to the coamplification of HER2 and TOP2A, as discussed in section 1.3. Topoisomerase IIα is an enzyme, found in the nuclei of mammalian cells, that regulates topological changes in DNA by introducing transient protein-bridged DNA breaks on both strands (Villman et al., 2002). Topoisomerase IIα is vital for many cellular processes including replication and transcription, and it is a known target of Anthracyclines (Villman et al., 2002). The binding of Anthracyclines causes the stabilization of DNA double stranded breaks and results in apoptosis (Villman et al., 2002). Many papers support the theory that Topoisomerase IIα is the target of Anthracyclines and the TOP2A gene should be used as a marker in its own right to predict the response of breast cancer patients to Anthracycline therapy (Jarvinen et al., 2003; Mano et al., 2007; Arpino et al., 2005; Tanner, 2006).

1.4.2.3. HER2 and targeted therapies

Trastuzumab (Herceptin) is the most widely used therapeutic option in breast cancer patients with HER2 gene amplification (Ross et al., 2009). Herceptin is a recombinant

human monoclonal antibody (Nahta and Esteva, 2006), that binds to the extracellular portion of the ErbB2 receptor (Ross et al., 2004). It was created by the insertion of murine antibody epitopes (clone4D5) into the framework of a consensus human IgG1 (Nahta and Esteva, 2006) (**Figure 6)**. The exact mechanism by which this antibody works is not entirely known (Ross et al., 2004; Nahta and Esteva, 2003). However, **Table 1** is a summarized list of the possible mechanisms of action, as compiled by Nahta and Esteva (2003, 2006). Herceptin was initially used to treat patients with HER2 amplification and metastatic breast cancer (Vogel et al., 2002). However, recent clinical trials have shown that it is effective as a first line therapy in conjunction with chemotherapy and surgery (Romond et al., 2005; Ross et al., 2009). Treatment of breast cancer with Herceptin has been shown to cause a 39–52% reduction in the recurrence rate and a 30% reduction in mortality, causing it to become widely accepted as the standard of care (Dinh et al., 2008).

In 2007, another targeted therapy, Lapatinib, was approved by the FDA to be used in HER2 positive metastatic breast cancer patients, in combination with Capecitabine (Ross et al., 2009). Lapatinib is an oral tyrosine kinase inhibitor that targets both HER2 and ErbB1 simultaneously (Moy and Goss, 2006). It works intracellularly by binding to the tyrosine kinase domain and competitively blocking ATP binding, thus halting subsequent downstream signalling (Moy and Goss, 2006). Lapatinib was approved seven years after Herceptin, therefore a great deal of clinical data, especially in the adjuvant setting, remains to be documented (Ross et al., 2009).

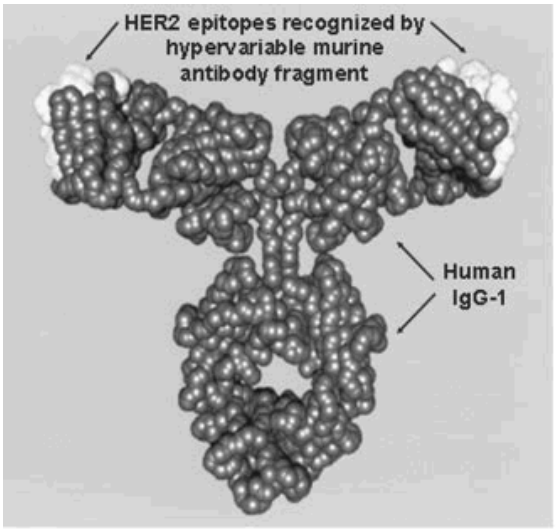

Figure 6: The Herceptin molecule includes the murine 4D5 antigen recognition antibody and the human IgG1 class antibody structure (Ross et al., 2004).

Table 1: Proposed mechanisms of action of Herceptin (Nahta and Esteva, 2003, 2006)

Herceptin proposed mechanisms of action
Internalization and degradation of the ErbB2 receptor: the drug disrupts receptor dimerisation and downstream signalling pathways
G_1 arrest and reduced cell proliferation: the drug induces $p27^{kip1}$-cdk2 complex formation and induces p27 levels
Apoptosis: the drug inhibits Akt activity
Suppresses angiogenesis: the drug reduces the tumour vasculature *in vivo*; reduces expression of the proangiogenic VEGF, TGF-α, Ang-1 and PAI-1 genes; induces the antiangiogenic TSP-1 gene.
Immune-mediated response: antibody-dependent cellular cytotoxicity; stimulates natural killer cells
Inhibits ErbB2 extra-cellular domain proteolysis

1.5. HER2 testing

International guidelines emphasize the necessity of testing every primary invasive breast cancer specimen for HER2 over-expression/gene amplification due to its value in determining both prognosis and treatment regimes (Wolff et al., 2007). Non-invasive tumours, with neoplastic proliferations of epithelial cells confined to breast ducts without basal membrane infiltration, are termed ductal carcinoma *in situ* (DCIS) and are not yet an indication for HER2 amplification testing (Wiechmann and Kuerer, 2008). At this point, no clinical trials have shown patients with DCIS to benefit from Herceptin, therefore only invasive breast carcinomas are recommended for HER2 testing (Ross et al., 2009). However, in the future, if anti-HER2 targeted therapies, such as Herceptin, are shown to reduce the development of HER2 positive DCIS into Invasive Carcinomas, then DCIS will become routinely tested for HER2 amplification as well (Ross et al., 2009).

In the clinical setting, HER2 is tested mostly by Immunohistochemistry (IHC) or Fluorescence *in situ* Hybridisation (FISH). Most laboratories perform these tests according to the guidelines proposed by The American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP). There are many other techniques by which HER2 amplification can be detected, including: Southern and slot blotting; RT-PCR; ELISA, and dimerization assays; all of which are beyond the scope of this project (Ross et al., 2009). IHC, FISH and their role in HER2 assessment are reviewed below.

1.5.1. Immunohistochemistry (IHC)

IHC detects protein over expression on the cell membranes. An antibody, labelled with a fluorescent dye such as fluorescein or rhodamine, binds to the desired protein (ErbB2 in this case), so that it can be localised within the cell and analysed under a light microscope (Strachan and Read, 2004). IHC allows for the identification of infiltrating carcinoma tumour cells, allowing these IHC results to be distinguished from those of the *in situ* cells (Ross et al., 2009).

The Dako Hercep Test and the Ventana Pathway are the two FDA approved, commercially-available IHC tests (Ross et al., 2009). According to the ASCO/CAP guidelines, a staining score of 0-1+ is considered negative, 2+ is equivocal, and 3+ is positive (Wolff et al., 2007). **Figure 7** below shows the four possible IHC staining patterns.

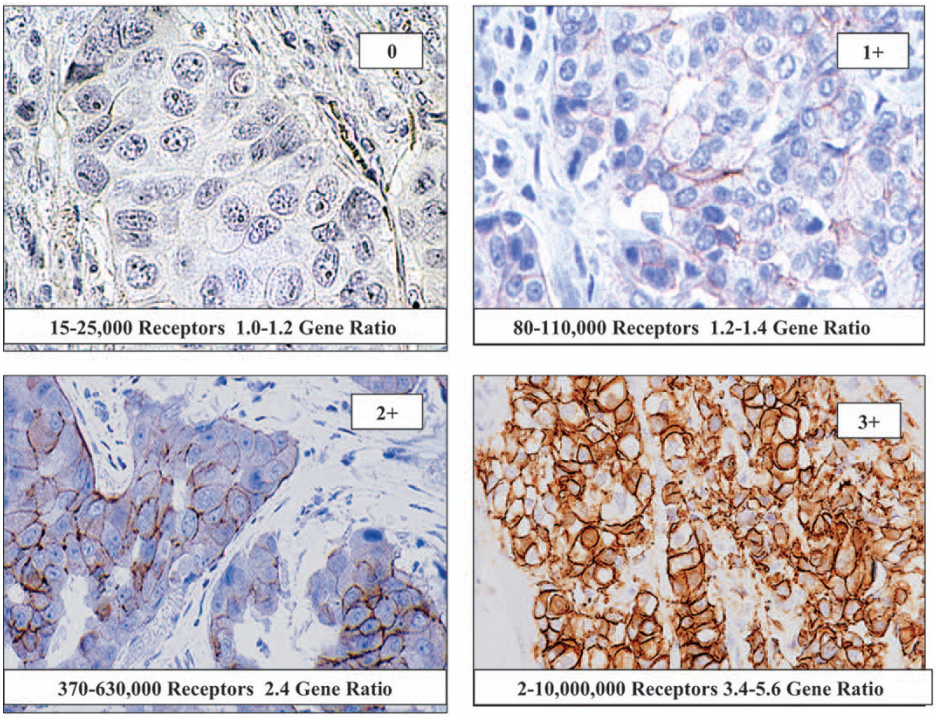

Figure 7: Four histological breast tumour sections showing the four classes of IHC scores and the number of receptors they represent. According to ASCO/CAP guidelines, 0 is defined as no membrane staining (Wolff et al., 2007). 1+ is defined as overall partial membrane staining, with complete membrane staining in less than 10% of the cells (Wolff et al, 2007). 2+ is defined as light to moderate complete membrane staining in more that 10 % of cells (Wolff et al., 2007). 3+ is defined as strong complete membrane staining in more than 10% of cells (Wolff et al., 2007). The figure also indicates the gene ratios (number of HER2 signals in relation to number of CEP17 signals), which would be calculated from FISH results (discussed below), from Ross et al., 2009.

1.5.2. Fluorescent *in situ* Hybridisation (FISH)

FISH is a technique in which a fluorescently labelled probe is hybridised to a chromosome region of interest, in this case the HER2 locus, and then scored under a fluorescent microscope. There are three FDA approved FISH tests currently available on the market: The first is the PathVysion HER2 DNA probe kit (Abbott Vysis, Inc.), which consists of two probes, one that hybridises to the HER2 gene (SpectrumOrange) and the other to chromosome 17 centromeric sequences (SpectrumGreen). This kit

detects amplification of the HER2 gene in relation to chromosome 17 centromere that act as an internal control (CEP17) (Wolff et al., 2006). The second kit is the INFORM HER2/neu Probe (Ventana, Inc.), which consists of only one probe that hybridises to the HER2 gene (Wolff et al., 2006). The third is PHarmDx (DAKO) which is also a dual probe and works the same as the PathVysion probe (Ross et al., 2009). A fourth commercially-available FISH probe, that is not FDA approved yet, is the Poseidon™ Repeat Free™ TOP2A (17q21), Her2 (17q12) & SE 17 Triple-Colour probe kit (Kreatech, Inc) that can detect HER2 and TOP2A gene amplification, in relation to CEP17, simultaneously.

According to the most recent ASCO/CAP guidelines (Wolff et al., 2007), a FISH result is considered positive if there are an average > 6 signals per cell nucleus when using the INFORM assay and when the HER2/CEP17 ratio is >2.2 with the PathVysion or PHarmDx assay (dual probe assays) (Wolff et al., 2007). A specimen that exhibits an average of 4-6 signals per nucleus or a HER2/CEP17 ratio of 1.8- 2.2 is considered equivocal. These patients still constitute a vague group that is poorly studied, and it is not yet clear whether or not these patients will benefit from Herceptin therapy (Wolff et al., 2007). A sample is considered negative with a HER2/CEP17 ratio of 1.8 or less, or if there are less than 4 signals per nucleus (Wolff, 2007). **Figure 8** shows an example of a normal HER2 positive and HER2 negative FISH result using the PathVysion kit.

FISH has been shown to be a superior technique to IHC in that: DNA is a more stable target than the HER protein, and it is less susceptible to variation caused by tissue handling (for example, variation in tissue fixation especially in regards to length of fixation, ethanol exposure, and antigen retrieval methods); additionally FISH is a quantitative interpretation and results in less subjectivity (concordance between observers is much higher than in IHC) (Hicks and Tubbs, 2005; Sauter et al., 2009).

Although FISH offers a very good HER2 amplification measurement, there are challenges to its interpretation in the clinical setting. First, there is the possibility of mistaking an *in situ* section of a tumour for the invasive one, resulting in a false positive if the DCIS section displays HER2 amplification while the invasive part does not (Ross et al., 2009). Although invasive tumours can arise from *in situ* tumours, the

rate of HER2 amplification is higher in DCIS than in Invasive Ductal Carcinomas (Ross et al., 2009; Latta et al., 2009). It has been proposed that the invasive component of a tumour arises from a clone of cells that develop invasive capabilities in the absence of HER2 amplification (Latta et al., 2002). This suggests that HER2 amplification is not a prerequisite for the progression of DCIS to Invasive Ductal Carcinomas, which would explain the higher rate of HER2 amplification in DCIS. (Latta et al., 2002).

Another area of controversy is chromosome 17 polysomy (Ross et al., 2009). Usually, polysomy 17 refers to there being 3 or more copies of chromosome 17 (indicated by increased CEP 17 signals) (Ross et al., 2009). However, there is no consensus on the exact definition of polysomy, especially regarding how many copies of a chromosome, and hence gene, would result in that gene's protein over-expression (Wolff et al., 2007). Some studies have shown that carcinomas with a high chromosome 17 polysomy and non-amplified HER2 gene, defined as a HER2/CEP17 ratio below 2.2, behave in the same way as HER2 negative tumours in regards to prognosis, mRNA expression and clinical features (Vanden Bempt et al., 2008; Torrisi et al., 2007; Dal Lago et al., 2006). However, other studies have shown that chromosome polysomy 17 can affect ErbB2 protein expression and polysomy 17 cases that also have a positive IHC test result should be considered for Herceptin treatment (Shah et al., 2009; Hoffman et al., 2008; Ma et al., 2005). Although not proven by large clinical trials, it appears that polysomy 17 patients that respond to Herceptin are restricted to those with a 3+ IHC score (Ross et al., 2009).

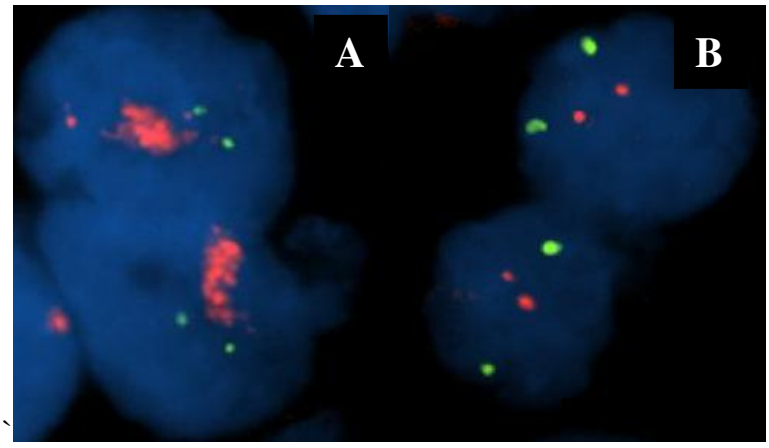

Figure 8: DAPI stained interphase nuclei hybridised with the Abbott-Vysis PathVysion dual colour probe. **A** shows a positive HER2 result, with many red signals representing HER2 and the two green signals representing CEP17. **B** shows a negative HER2 result with only two copies of HER2 and two CEP 17 signals.

1.6. Current challenges experienced with the commercially-available HER2 FISH probe kits

Other than the difficulty of distinguishing *in situ* cells from invasive ones, and the potential confusion caused by polysomy, another major challenge to FISH testing using the dual-colour probe kits, such as the PathVysion, is the emergence of cases that appear to exhibit amplification of the chromosome 17 control (CEP17) (Jacobson et al., 2004; Troxell et al., 2006; Press, 2006). The appearance of increased CEP 17 signals can make the interpretation of FISH data in accordance with international guidelines complex and ambiguous. In our laboratory at the Charlotte Maxeke Johannesburg Academic Hospital, Johannesburg, these ambiguous cases are observed at a rate of 1.2% (CEP17 signal> 9). The problem is that the interpretation of the FISH data can result in a HER2/CEP17 ratio < 2.2, which is considered negative, when it is possible that the HER2 gene is in fact amplified (together with CEP 17). Patients displaying these aberrant FISH signals could potentially be deprived of Herceptin, which may dramatically improve their chances of survival.

As advocated by Sauter et al. (2009), an internal probe on chromosome 17, but not in the HER2 amplicon is essential in HER2 assessment (Sauter et al., 2009). This internal control has two functions: the first is to assess successful hybridisation in order to determine which nuclei can be scored; the second reason is to differentiate specimens with chromosome 17 polysomy from those with true HER2 amplification (Sauter et al., 2009). As previously mentioned, there is no consensus on the exact definition of polysomy (Wolff et al., 2007). According to ASCO/CAP guidelines, when using single-colour probes, a HER2 copy number > 6 is considered positive. It is here that we encounter the problem of what can be designated ambiguous, with potential CEP17 amplification. To be consistent one would expect 7 or more copies of CEP17 to be considered amplification of the centromeric region. However, some studies have shown that specimens with polysomy, even with more that six copies of

CEP17, behave in the same way as HER2 negative specimens (Vanden Bempt et al., 2008; Dal Lago et al., 2006). In another study by Jacobson et al., (2004) they only excluded samples from their cohort with more than 10 copies of CEP17 due to suspected centromeric amplification (Jacobson et al., 2004). This shows that they still considered any chromosome 17 copy number below this to be polysomy (Jacobson et al., 2004). Specimens with high polysomy (> 6 copies of CEP17) and IHC scores of 2+ have also not yet been shown to benefit from Herceptin.

In our laboratory, cases (both lobular and ductal, in situ and invasive carcinoma) considered ambiguous were those that presented with: 1) innumerable clusters of red and green signals; or 2) green signals that are too numerous to be accounted for by 17 polysomy (**Figure 9**). Any CEP17 signal > 6 was considered ambiguous, however only specimens with CEP17 signals > 9 were included in this study based on those of Van Den Bempt (2008) and Jacobson et al. (2004).

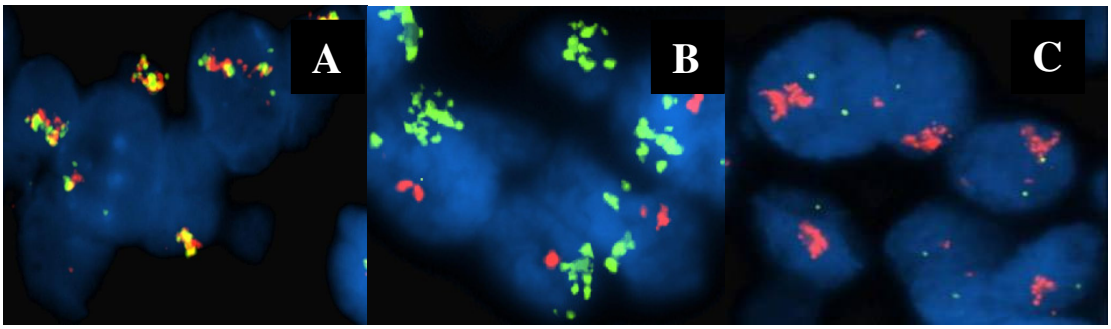

Figure 9: DAPI stained interphase nuclei hybridised with the Abbott-Vysis PathVysion dual colour probe showing ambiguous cases **A** with innumerable red and green signals (resulting in yellow masses), and **B** with green (CEP17) signals that are too numerous to be accounted for by chromosome 17 polysomy. In comparison to a normal positive result (**C**), it is clear that these cases are not displaying the expected HER2/CEP17 patterns.

2. AIMS OF THE STUDY

The overall objective of the study was to investigate the ambiguous cases of infiltrating breast carcinoma described above, using the FISH technique, in order to both clarify the status of the HER2 gene and to propose a more effective control. This main objective was divided into three specific objectives:

AIM 1:

Based on the high number of green (CEP17) signals seen in ambiguous cases, it was hypothesized that the chromosome 17 control was being amplified. Therefore, the first aim of the study was to confirm CEP17 amplification in these ambiguous cases and show that CEP17 is no longer an adequate internal control in FISH HER2 testing. For this reason, despite there being many ambiguous cases (CEP17 > 6), only those with more than nine copies of CEP17 were included, in order to show CEP17 amplification unequivocally.

AIM 2:

If CEP 17 was shown to be amplified in aim one, the next aim was to develop an in-house FISH probe that would act as a superior control to CEP17 in HER2 testing. Once developed, this probe could be tested on ambiguous cases. The advantage of such a control is the reduction of ambiguity in the interpretation of FISH signals, caused by CEP17 amplification, and a more accurate determination of HER2 amplification in line with international guidelines.

AIM 3:

A review of the literature showed that the HER2 amplicon is a dynamic one that harbours many genes, such as TOP2A and RARA that potentially play a role in determining the treatment of breast cancer patients. Therefore the third aim was to further elucidate the components of the HER2 amplicon by using FISH probes to determine TOP2A and RARA amplification in these ambiguous cases.

While the main objective of the project was to investigate ambiguous cases, a further aim was included that was carried out concurrently to the main project. This aim was to analyse the rates and trends of HER2 positivity in breast cancer samples (both invasive and *in situ* , ductal and lobular carcinomas) submitted to our laboratory, the Somatic Cell Genetics unit, at the Charlotte Maxeke Johannesburg Academic Hospital for routine FISH HER2 testing.

3. MATERIALS AND METHODS

3.1. Analysis of HER2 positivity in breast cancer samples submitted to our laboratory at Charlotte Maxeke Johannesburg Academic Hospital for routine FISH testing

During the project, 1558 breast cancer specimens were sent for FISH routine testing of HER2 amplification to our laboratory, Somatic Cell Genetics unit, Charlotte Maxeke Johannesburg Academic Hospital, Johannesburg, South Africa. These specimens included both invasive (the majority) and in situ ductal and lobular carcinomas. The patient results, along with demographic information, were captured into a database. These data were analysed and used to: 1) Determine the rate of HER2 amplification in our laboratory cohort; 2) Compare the rate of HER2 amplification between African and Caucasian South African women; 3) Compare the rate of HER2 amplification between African and Caucasian South African women with early-onset (≤ 35 years old) breast cancer; 4) Compare the incidence of early onset breast cancer between the two ethnic groups. To allow for simplicity, women of Indian and Mixed ancestry were included in the Caucasian group.

3.1.1. Statistical analysis

The rates of HER2 amplification, as well as the incidence of early onset breast cancer were compared between the two ethnic groups using the Chi-squared test.

3.2. Investigation of ambiguous cases by Fluorescent *in situ* Hybridisation with four different FISH probe kits

Fluorescent *in situ* hybridisation is an effective technique that allows for the detection of copy number changes in genes known to be associated with cancer. FISH involves the denaturation of double-stranded probes and target DNA, and then the hybridisation of the fluorescently labelled probe to the target DNA.

In FISH, a DNA probe can be directly labelled, by the inclusion of fluorescently labelled nucleotides, or indirectly labelled, by the inclusion of nucleotides containing reporter molecules which are then bound to a fluorescently labelled affinity molecule. (Strachan and Read, 2004)

3.2.1. Sample Collection

Ethical clearance was obtained from the University of the Witwatersrand Ethics Committee. (Clearance certificate number M090476, subproject of M050551).

Breast cancer specimens for this study were identified during routine HER2 FISH testing in our laboratory. Cases were considered ambiguous if they presented with 1) innumerable clusters of red and green signals; or 2) green signals too numerous to be accounted for by chromosome 17 polysomy (i.e. more than 6 CEP17 signals). Although any case with more than 6 CEP17 signals was considered ambiguous, for the purpose of this study, which was to show CEP17 amplification unequivocally, only 19 ambiguous cases, which all had more than 9 CEP17 FISH signals, were included. Of the 19 ambiguous cases included in this study: 9 were infiltrating ductal carcinoma; 1 was a ductal carcinoma *in situ;* and in the remaining 9 the specific neoplasm was not included in the patient report and could thus not be determined. The original slides and formalin-fixed paraffin embedded (FFPE) tissue blocks of the included samples were collected at the time of routine testing. Three micron sections were cut from the FFPE blocks and mounted onto slides for FISH preparation. The H&E slides (made if not available) were reviewed by an experienced pathologist and areas containing 80% of tumour cells were circled.

3.2.2. FISH probes description

3.2.2.1. PathVysion HER-2 DNA probe kit

Ambiguous cases were first routinely tested with the PathVysion HER-2 DNA probe kit (Abbot Vysis Inc). This dual-colour probe kit has probes that hybridise to the

HER2 gene (SpectrumOrange®) and to chromosome 17 centromere (CEP17) (SpectrumGreen®), which acts as an internal control.

3.2.2.2. Poseidon Repeat Free TOP2A (17q21), Her2 (17q12) & SE 17 Triple-Colour probe kit

Ambiguous cases were re-tested with the commercially- available Poseidon Repeat Free TOP2A (17q21) & Her2 (17q12) & SE 17 Triple-Colour probe kit that can simultaneously detect HER2 and TOP2A amplification by including probes for the HER2 gene (red fluorochrome), chromosome 17 centromere (CEP17/SE17) (blue fluorochrome) and the TOP2A gene (green fluorochrome) (**Figure 10**). This was to determine whether aberrant FISH signals seen in the ambiguous cases during routine testing were an artefact of the PathVysion probe, or true centromeric amplification. It also allowed for the assessment of TOP2A amplification.

3.2.2.3. In-house probe kit

After testing samples with the Poseidon probe, an in-house probe kit, with a control potentially superior to CEP17, was designed and produced. In-house FISH probes are prepared using appropriate Bacterial Artificial Chromosomes (BAC) and the Nick Translation dual colour labelling method.

3.2.2.4. Vysis LSI PML/RARA Dual Colour, Dual Fusion Translocation Probe kit

To assess the status of the RARA gene in ambiguous cases, samples were hybridised with Vysis LSI PML/RARA Dual Colour, Dual Fusion Translocation Probe kit that includes probes for the genes PML, on chromosome 15 (SpectrumOrange) and RARA (SpectrumGreen) (**Figure 10**). This probe is usually used in the diagnosis of Acute Promyelocytic Leukaemia. In this instance it allowed for the detection of RARA amplification, while PML acted as a control for hybridisation

3.2.3. Design, production and testing of the in-house probe cocktail

3.2.3.1. Probe design

From a number of genes that were investigated as potential controls, ACTG1 was selected for the following reasons:

1) It is located at 17q25.3, 416 megabases telomeric to the HER2 gene and unlikely to be included in the HER2 amplicon due to the large distance between the two genes. **(Figure 10)**.
2) It is a highly conserved, housekeeping gene (Eisenberg and Levanon, 2003; Rubenstein, 1993).
3) A review of genetic websites and an investigation of articles on Pubmed indicated that it plays no known role in breast or other cancer formation and is thus more likely to remain stable and unaltered.

Ensembl was used to find the BAC clones, RP11-730A09 and RP11-94L15, that cover the ACTG1 and HER2 regions respectively. The BAC clones were ordered from the BACPAC resource centre, Children's Hospital Oakland Research Institute, CA, USA. BACS are recombinant DNA vectors, based on the *E-coli* fertility plasmid, F-factor, that can be transferred into *E-coli* by electroporation to allow for replication.

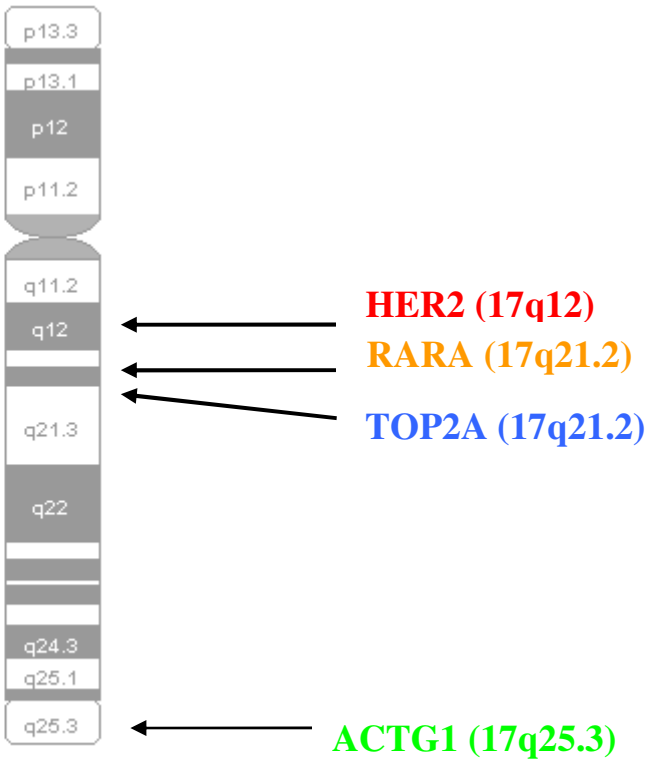

Figure 10: Ideogram of chromosome 17 showing the layout of FISH probes for the genes investigated in this study.

3.2.3.2. Culturing of bacterial cells for BAC DNA extraction

Escherichia coli (*E-coli*), containing the HER2 and ACTG1 BACS, were received from the BACPAC resource centre in agar stabs. The *E-coli* cells were inoculated in 5 ml of sterile Luria-Bertani (LB) broth (Appendix A) with the antibiotic Chloramphenicol (Sigma®) (20ug/ml, dissolved in 100% ethanol) and grown overnight at 37°C in an orbital incubator at ± 200 rpm. The following day, cell stocks were made by the addition of 850 µl of culture to 150 µl of Glycerol solution (Appendix A) and stored at -70°C.

Agar plates (Appendix A) containing Chloramphenicol were streaked with the remaining culture and incubated at 37°C overnight. From these, single bacterial colonies were selected and inoculated in 5 ml LB broth with 20ug/ml Chloramphenicol and grown overnight at 37°C in an orbital incubator at ± 200 rpm. Cell stocks were made and stored as described above. The remainder of the culture

was inoculated into 200 ml LB broth with 20ug/ml Chloramphenicol and grown overnight at 37°C in an orbital incubator at ± 180 rpm until *E-coli* cells reached the log phase of growth. Cultures were centrifuged for 15 minutes at 5000 rpms in 50 ml Nunc tubes, supernatant was discarded and pellets were stored at – 70°C until DNA extraction. All inoculation was performed under sterile conditions.

3.2.3.3. DNA isolation from BAC clones

BAC DNA was extracted using the Qiagen® Plasmid Purification Midi kit and carried out according to the manufacturer's instructions. Pellets were re-suspended in 4 ml resuspension buffer, and cells were lysed by the addition of 4 ml of lysis buffer. Tubes were gently inverted to mix contents and incubated at room T° for 5 minutes. The lysis reaction was neutralized by the addition of 4 ml of chilled neutralisation buffer. Samples were incubated on ice for 15 minutes to allow for precipitation of genomic DNA and cellular debris and centrifuged at 13000 rpm for 30 minutes at 4ºC. The supernatant, containing plasmid DNA, was then further centrifuged at 13000 rpm for 15 minutes at 4ºC to pellet the remaining precipitate. The DNA-containing supernatant was applied to a Qiagen-tip 100 that had been previously equilibrated by the addition 4 ml of equilibration buffer to it. For the ACTG1 DNA, yields were initially low; therefore, the supernatant from two lysis reactions was applied to one column. The DNA-containing column was washed twice, by the addition of 10 ml wash buffer to the column. DNA was eluted into a new tube with 5 ml of heated (65ºC) elution buffer. DNA was precipitated by the addition of 3.5 ml isopropanol, rinsed in 2 ml of 70 % ethanol (room T°) and air-dried. Pellets were re-suspended in 100 µl of 1 X TE buffer (Promega®) and dissolved overnight on a platform shaker.

3.2.3.4. Estimation of DNA yields

BAC DNA quantities were determined by gel electrophoresis on 2 % agarose gels (Appendix A). The band intensity of 2 µl of BAC DNA samples was visually compared to that of Lambda DNA (250 ng/µl, Boehringer Mannheim) of various known concentrations that were electrophoresed alongside the samples. DNA with a concentration ≥ 50 ng/µl was used for the nick-translation labelling.

3.2.3.5. Probe labelling

BAC DNA was directly labelled by the nick translation method. In this technique, DNase1 makes single strand breaks (nicks) in double stranded DNA. DNA Polymerase 1, using its endonuclease activity, then adds new (some fluorescently labelled) nucleotides, to the free 3' hydroxyl group, while removing nucleotides 5' to 3' by its exonuclease activity (Strachan and Read, 2004). This results in a collection of fluorescently labelled DNA fragments representing the probe that can be hybridised to the gene of interest.

The nick translation labelling reaction contained:

- 1µg template DNA
- 10 µl 10 X nick-translation buffer (Appendix A)
- 10 µl 0.1 M β-mercaptoethanol (Appendix A)
- 8 µl dNTP mix (Appendix A)
- 3µl 10u/µl DNA Polymerase 1 (Promega)
- 1 µl DNAse 1 (dilution of stock DNAse 1 (Appendix A) 2:1000 in distilled water)
- H_2O to a final volume of 100 µl.

Differing DNase1 dilutions were tested on DNA before the labelling reaction to establish the optimal concentration that would result in 200-500 bp fragments. Fragments of this size are small enough to penetrate the nuclear pores, but are large enough to avoid cross-hybridisation, and are thus considered suitable for FISH. The labelling reaction was incubated at 15°C for 2 hrs in an Eppendorf thermal cycler. The ACTG1 DNA was labelled with Spectrum green-dUTP (Abbot Vysis Inc), and the HER2 DNA was labelled with Spectrum orange-dUTP (Abbott Vysis Inc.) After the two hour incubation, 8 µl nick translation mix was denatured for 3 minutes at 93ºC and run on a 2% agarose gel alongside a 100 bp DNA ladder (Fermentas®) in order to determine if the probe digestion was between 200-500 bps. The probe was further digested (subsequent to the addition of another 1 µl of DNase1 dilution) at 15ºC if probe fragments were too large.

After digestion, the DNAse 1 enzyme was then inactivated by the addition 3 µl 0.5M EDTA and 1 µl 10% SDS to the nick translation reaction, followed by heating at 60ºC for 15 minutes. The DNA (probe) was then precipitated by the addition of 10 % of total volume 3M NaAc3 (Merck®), 2.5x ice cold EtOH and 20 µg of Cot1 Human DNA (Invitrogen®), placed at -70º C overnight. Cot1 DNA binds to repetitive DNA sequences and allows for unique sequence probe hybridisation. Cot1 DNA concentration was increased to 30 µg when making the HER2 probe, due to detected non-specific binding. The following day, the precipitation mix was centrifuged at 13000 rpms for 30 minutes at 4 ºC. The supernatant was discarded and the pellet was rinsed in 200 µl ice cold 70% EtOH, centrifuged at 13000 rpms for 10 minutes at 4 ºC. Pellets were air-dried, resuspended in 50 µl Hybridisation buffer and allowed to dissolve overnight on a platform shaker.

3.2.3.6. Verification of ACTG1 and HER2 probe hybridisation on control metaphases

The ACTG1 and HER2 in-house probes were tested on control metaphases, to determine if they were hybridising to the correct chromosomal regions, and that there was no cross hybridisation onto other chromosomes. This would be as a result of contamination with other DNA during inoculation of BAC clones or insufficient blocking of repetitive sequences. Every newly produced probe was tested in this way before being hybridised to samples as part of verifying the probe specificity.

3.2.3.6.1. Peripheral Blood Cell Culturing

Unsynchronised peripheral blood cultures were established as a source of control metaphases for verification of ACTG1 and HER2 probe hybridisation specificity. Fresh blood was collected from a healthy volunteer and 0.5 ml of blood was added to 4.5 ml complete medium (Appendix A). Cell division was stimulated by the addition of 100 µl of Phytohaemaglutinine (PHA). The cell cultures were incubated overnight at 37°C**.**

3.2.3.6.2. Harvesting of cell cultures

Mitotic arrest was performed by the addition of 100 µl colcemid to each 5 ml blood culture, followed by further incubation at 37°C for 30 minutes. Cultures were then transferred to 15 ml tubes and centrifuged at 1000 rpm for 8 minutes. The supernatant was removed and 6 ml of hypotonic solution (0.075M prewarmed KCl) was added to the pellet, which was mixed and followed by incubation at 37°C for 20 minutes. Thereafter cells were prefixed with 7-10 drops of ice-cold fixative (3:1 Methanol: Acetic acid). The mixture was centrifuged at 1000 rpm for 8 minutes, the supernatant was discarded and cell pellets were fixed with 6 ml fixative. This was repeated 3-5 times.

3.2.3.6.3. Slide making

Microscope slides were stored in methanol and polished with folded tissue before slide making. Slides were first flooded with 3:1, Methanol:Acetic Acid fixative. The cell pellet was resuspended in a small volume of fixative and 50 µl of the cell suspension was dropped onto the slides from a height of ±20cm. Slides were allowed to dry and then dehydrated in series of 70%, 90% and 100% alcohol for 5 minutes each and aged overnight at room temperature.

3.2.3.7. Validation of the in-house probe kit on previously established HER2 positive and negative samples

Based on results from the ambiguous cases, the HER2/ACTG1 in-house probe cocktail was tested on 20 negative HER2 and 20 positive HER2 samples (as determined by the PathVysion kit in routine testing). 11 of these controls were infiltrating ductal carcinomas, while the rest were unknown due to lack of specification of histological type on patient report. Although this was a small sample set, it would allow for some preliminary data on whether the cocktail could be used commercially in the clinical setting.

3.2.4. Preparation of slides for FISH

Three micrometer tissue sections on positively charged slides were first baked overnight at 60°C. They were then deparaffinized by three successive washes of 15 minutes each in xylene (Merck®). The slides were then dehydrated twice in 100% alcohol for 5 minutes. The acid pre-treatment, which weakens tissue so that it can be digested optimally, was performed by placing the slides in 0.2 HCl for 20 minutes followed by two rinses in double distilled H_2O for 3 minutes each, and a 2X SSC (Appendix A) wash for 3 minutes. All of the above steps, except for the Xylene (in fume cabinet) were done at room temperature on a shaking platform. Slides were then allowed to dry on a 37ºC heating block.

To remove crosslinks, which occur in the DNA due to the formalin fixed paraffanisation process, slides were placed in 1M NaSCN (Appendix A) at 80ºC for 30 minutes. Thereafter, slides were placed in dH_2O for 1 min, followed by two rinses in 2X SSC for 5 minutes. These steps were all carried out at room Tº on a shaking platform.

The pepsin treatment, which removes membrane proteins to allow for probe penetration, was done by placing the slides in 50 ml 0.1N HCl with 25mg Pepsin (Roche®), immersed in a waterbath at 37ºC. Slides were placed in the pepsin solution for 20 minutes-1hour, until the tissue was adequately digested. Thereafter slides were rinsed twice in 2X SSC for 5 minutes and then allowed to dry on a 37ºC heating block.

Slides were placed in 1% Formaldehyde (Appendix A) at room Tº shaking for 10 minutes in order to refix tissue loosened by the pepsin solution. This was followed by two rinses in 2X SSC for 5 minutes.

Due to limited tumour specimen and FISH slides, slides from the Poseidon probe were reused for the RARA assessment. Coverslips were removed from the slides which were then washed in a series of 70%, 90%, 100% alcohol prior to hybridisation with the RARA probe.

3.2.5. Preparation of probes

The PathVysion and Poseidon probes were pre-prepared by the manufacturer and applied to the slides straight from the commercial vial. For the in-house probe, 10 µl (200 ng of probe DNA) of ACTG1 probe and 10 µl (200ng of probe DNA) of HER2 probe were combined. The PML/RARA probe mixture contained 7 µl hybridisation buffer (included in probe kit), 2 µl sterile H_2O and 1 µl of probe.

3.2.6. Denaturation of probe and target DNA

For the PML/RARA and the in-house probe kit, slides were placed in denaturing solution (Appendix A) at 75ºC for 5 minutes and then dehydrated in ice cold 70%, 90%, 100% alcohol for 5 minutes each. The denaturing solution contained formamide, which chemically disrupts the hydrogen bonds between DNA strands and destabilises the duplex, resulting in the lowering of the DNA melting temperature. Probes were denatured by placing them in a 75ºC waterbath for 5 minutes.

For the PathVysion and Poseidon probes, 10 µl of probe was applied to a coverslip which was then placed onto the slide and sealed with rubber cement. Slides were placed in a Thermobrite™ (Abbot Molecular), as optimised by the manufacturer, overnight on a programme that started with a denaturation step (95ºC for 5 minutes) followed by a hybridisation step at 37°C for 24 hours. For all the probes, where tumour regions were small, coverslips were cut in half and 5 µl (10 µl for the in-house probe) of probe was applied.

3.2.7. Hybridisation of the in-house and RARA probes

For the RARA and in-house probes, slides were air-dried and 10 µl and 20 µl of probe was applied to slides respectively, which were sealed with rubber cement (Marabu®) and placed in a humidity chamber in the incubator to hybridize at 37°C overnight.

3.2.8. Post hybridisation washing

In order to remove unhybridised probe, slides were washed in a solution of 2X SSC with 0.05% Tween20 (Merck®) at 73ºC for 4 minutes. They were then counterstained in 4',6-Diamidino-2-phenylindole (DAPI) solution (0.2 mg/ml stock (Appendix A) in 2 X SSC) for 15 minutes (shaking at room Tº), and rinsed for 2 minutes at room Tº in 2X SSC and 0.05% Tween20. Coverslips were mounted onto slides using Vectashield anti-fade solution (Vecta Laboratories®).

3.2.9. FISH analysis and interpretation

All samples were analyzed under a fluorescent Olympus BX61 microscope. Images were captured using the Genus™ Imaging system. Signals in 60 nuclei, in regions with ≥ 80% invasive tumour cells were manually counted and recorded (100 cells were counted for the PathVysion probe due to routine/diagnostic purposes). Ratios from routine cases had already been determined (average number of red signals (HER2)/ average number of green signals (CEP17)). For the Poseidon and in-house probe, a ratio was determined for each nucleus and a mean ratio calculated. TOP2A amplification was determined as an absolute value per cell (due to amplification of the centromere, it was not possible to used centromeric signals as a reference). According to ASCO/CAP guidelines, an absolute HER2 gene copy number greater than six in single-probe kits is considered positive, therefore TOP2A signals > 6 were also considered positive. RARA amplification was also determined as an absolute value per cell, with >6 signals considered positive. For every slide, in addition to counting 60 nuclei, the whole slide was scanned to obtain a general signal pattern and to identify any other aberrant FISH patterns not previously seen.

The common signal patterns from the PathVysion, Poseidon and In-house probes were recorded and compared. Ratios (HER2/Control probe) from these three probes were also calculated and compared. The Poseidon and PML/RARA probes were used to determine TOP2A and RARA amplification respectively in ambiguous cases.

3.2.9.1. Statistical Analysis

Ratios obtained from the three different probes (PathVysion; Poseidon; In-house cocktail) were compared with three matched-pair t-tests, using SAS enterprise guide computer software.

RESULTS

4.1. Analysis of HER2 positivity in breast cancer samples submitted to our laboratory at Charlotte Maxeke Johannesburg Academic Hospital for routine testing

1558 breast cancer specimens were routinely tested for HER2 amplification at our laboratory during the length of this project. These patient results, along with demographic information were entered into a database and used to determine the following trends:

Table 2: Rate of HER2 amplification in the South African population

HER2 amplification status	n	%
Positive	683	44%
Negative	875	56%
Total	1558	100%

It is clear that the rate of HER2 amplification in this cohort was higher than the general rate of HER2 amplification (20-30%) due to referral bias (see Discussion).

Table 3: Rate of HER2 amplification in African vs. Caucasian South African women

	HER2 amplification Status	n	%
African (360 samples)	Positive	188	52%
	Negative	172	48%
Caucasian (1198 samples)	Positive	510	43%
	Negative	688	57%

The rate of HER2 amplification is significantly higher ($p < 0.05$) amongst African patients (52%) than amongst Caucasian patients (43%).

Table 4: Rate of HER2 amplification in early onset (≤ 35 years) breast cancer amongst African and Caucasian South African women

	HER2 amplification Status	n	%
African (35 samples)	Positive	17	49%
	Negative	18	51%
Caucasian (25 samples)	Positive	17	68%
	Negative	8	32%

In the early onset cohort three trends were observed:

1) In African women, the rate of HER2 amplification in the younger group (49%) is similar to that of the general African group (52%)
2) In Caucasian women, the rate of HER amplification in the younger group (68%) is significantly higher ($p < 0.05$) than the general group (43%) (**Table 3**).
3) The incidence of early onset breast cancer is significantly higher ($p < 0.05$) amongst African patients (35 out of 360 samples) than amongst Caucasian patients (25 out of 1198 samples).

4.2. Investigation of ambiguous HER2 amplification cases by Fluorescence *in situ* Hybridisation with four different FISH probe kits

4.2.1. Verification of in-house probe hybridisation on control metaphases

Prior to usage on ambiguous cases, the in-house probes for the genes ACTG1 and HER2 were hybridised to control metaphases to ensure hybridisation to the correct region, and to ensure that there was no cross hybridisation to other chromosomal regions (**Figure 11**). The ACTG1 probe hybridized to the correct region, 17q25.3; the fluorescent signal was clear and bright, with no signs of cross hybridisation. The in-house HER2 probe was also correctly hybridising to 17q12. The fluorescent signal of this probe was clear, bright and annealing to the correct region.

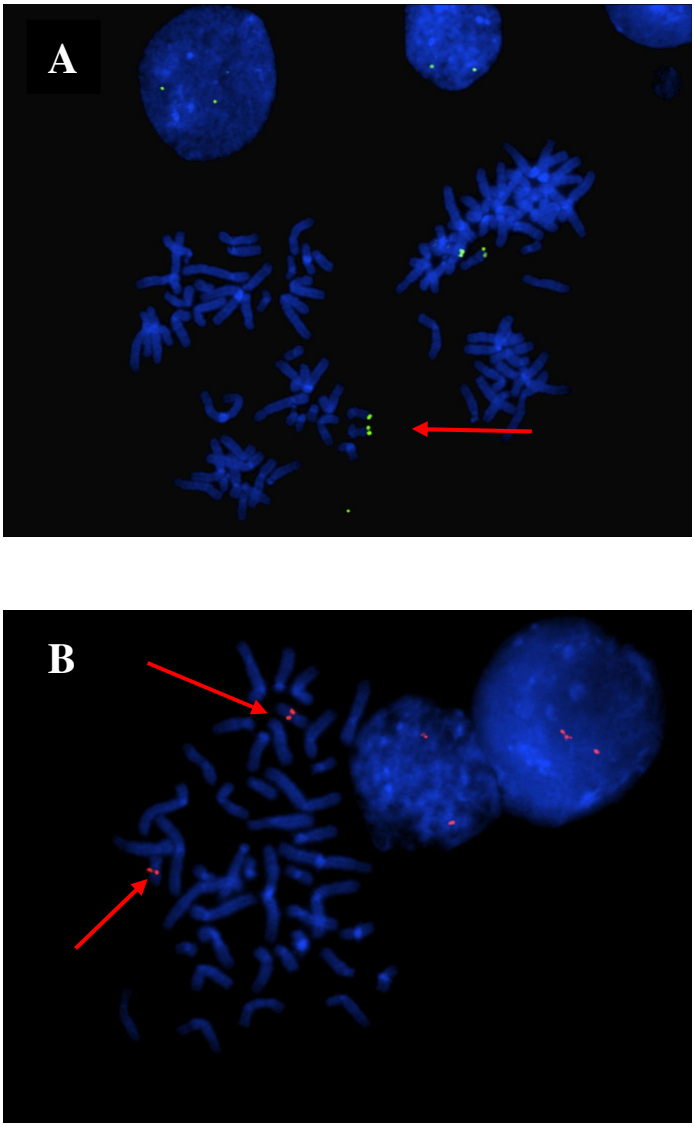

Figure 11: DAPI-stained control metaphases hybridised with in-house probes. **A** shows ACTG1 (labelled with SpectrumGreen) hybridising to 17q25.3 on a normal metaphase. **B** shows HER2 (labelled with SpectrumOrange) hybridising to 17q12 on a normal metaphase. For both probes, two fluorescent signals can also be clearly seen in interphase nuclei.

4.2.2. Results from the PathVysion, Poseidon, In-house and PML/RARA probe kits hybridised to ambiguous cases

The use of four FISH probe kits allowed for the identification and investigation of centromeric amplification in ambiguous cases (PathVysion and Poseidon probes), as well as for the assessment of TOP2A and RARA amplification (Poseidon and PML/RARA probes). It also allowed for the assessment of the in-house kit containing a superior control to CEP17 on ambiguous cases.

A summary of the common signal patterns observed with each of the four probe cocktails, hybridised to the 19 ambiguous cases can be seen in **Table 5**. The signal patterns were used to determine the components of the HER2 amplicon for each ambiguous case **(Table 5).** Of the 19 ambiguous cases, 16 had amplicons that included the centromere. This was determined by amplified CEP 17 signals seen in both the PathVysion and Poseidon probes. Of the remaining three: one (case 16), that had what appeared to be centromeric amplification, was in fact due to artefact/background. Another (case 17) only had high polysomy (4-6 CEP 17 signals with Poseidon probe) or potential artefact (5-10 CEP17 signals with PathVysion probe) with HER2 amplification. The final ambiguous case (case 13), also had polysomy (2-4 CEP17 signals with Poseidon probe), without HER2 or centromeric amplification. However, the number of ACTG1 signals seen in case 13 was higher than those of HER2, which was not amplified. This was not an expected result, and suggested that there may be amplification occurring in this region. Two of the ambiguous cases (case 15 and case 19) had centromeric amplification only, without any HER2 amplification (**Figure 12).**

To further demonstrate centromeric amplification and show the superiority of ACTG1 as a control to CEP17, the HER2/control ratios from the PathVysion, Poseidon and In-house probes were calculated and compared (**Table 6).** Most of the ambiguous cases with centromeric amplification had HER2/CEP17 ratios that would result in them being classified as negative (according to international guidelines), yet became positive (ratio >2.2) when assessed using the HER2/ACTG1 ratio. The statistical analysis showed that ratios from the in-house probe were significantly higher than those of the two commercial probes, $p < 0.05$. Visual comparison of the images from

the three probes showed that HER2 amplification was clearer when using the ACTG1 control (**Figure 13).**

Six of the 19 cases (cases 2, 3, 5, 14, 18) had amplicons that included TOP2A. All of these cases were HER2 positive and all also included centromeric amplification. Five of the 6 cases with TOP2A amplification also displayed RARA amplification **(Figure 14).** The exception was case 18 where RARA hybridisation was unsatisfactory and therefore uninformative. In case 1, TOP2A hybridisation was unsatisfactory and therefore uninformative. In case 4, TOP2A and RARA probes displayed unsatisfactory hybridisation, therefore no conclusions could be made regarding the amplification of these genes. The same was seen in case 6 for the RARA probe.

Table 5: Summary of common signal patterns obtained from the four probe cocktails as well as a the proposed amplicon for each ambiguous case

Case	PathVysion Probe	Poseidon Probe	In-house Cocktail	PML/RARA probe	Proposed amplicon
1	>20r; 15g	>20rb; 0-1g	>20r; 1-2g	2-4r 2-4g	HER2 ; CEP17 (TOP2A uninformative)
2	>20r; 4-10g	>20rbg	>20r; 1-2g	>20r 2-4g	HER2; CEP17; TOP2A; RARA
3	>20r; 15g	>20rbg	>20r; 2-5g	>20r 2 g	HER2; CEP17; TOP2A; RARA
4	>20rg (clone)	>20rb; 0-2g	>20r; 1-2g	-	HER2; CEP17 (TOP2A and RARA uninformative)
5	>20rg	>20rbg	>20r; 1-2g	>20r 2 g	HER2; CEP17; TOP2A; RARA
6	>20r;10-20g	>20rb; 1-3g	>20r; 2-3g	-	HER2; CEP17 (RARA uninformative)
7	>20rg	>20rb; 1-4g	>20r; 2-3g	1-2r 1-2g	HER2, CEP17
8	>20rg	>20rbg	>20r; 2-4g	>20r 3-6g	HER2; CEP17; TOP2A; RARA
9	>20rg	>20r;1-4g; 4-20b	>20r; 3-8g	3-4r 3-4g	HER2;CEP17
10	>20rg	>20r;2-4g; 4-20b	>20r:2g	2r 2g	HER2; CEP17
11	>20rg	>20rb, 2-4g	>20r; 2-5g	3-4r 3-4g	HER2; CEP17
12	>20rg (clone)	>20r; 2-3g;>20b	>20r; 2-3g	2-4r 2-4g	HER2;CEP17
13	3-15r; 1-10g	2-7r; 2-5g;2-4b	2-4r; 4-11g	2-4r 2-4g	Unknown
14	>20rg	>20rbg	15-20r;3-4g	12-15r 2-4g	HER2; CEP17; TOP2A; RARA
15	2-6r; 15-20g	2-4r; >20b;1-3g	3-6r; 1-3g	3-4r 3-4g	CEP17
16	2r; 2-10g	2rgb	1-2r; 1-2g	2r 2g	No amplification
17	>20r; 5-10g	>20r; 2g;4-6b	>20r;1-2g	`1-2r 2g	HER2
18	>20rg	>20rb; 4-12g	>20r 2-3g	-	HER2, CEP17; TOP2A; (RARA uninformative)
19	2r >20g	2-3r >20b 2-3g	2-3r; 2-3g	2r 2g	CEP17

PathVysion probe: r = HER2 signal; g = CEP17 signal

Poseidon probe: r = HER2 signal; b = CEP17 signal; g = TOP2A signal

In-house probe: r = HER2 signal; g = ACTG1 signal

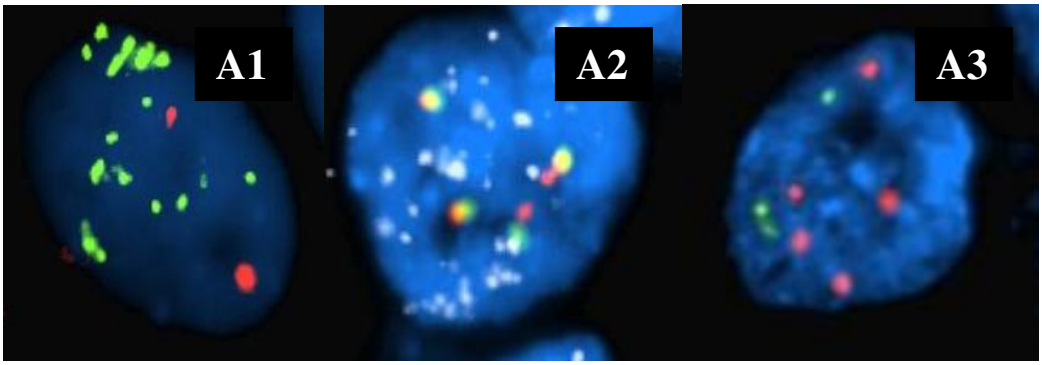

Figure 12: DAPI stained interphase nuclei hybridised with: the Abbott-Vysis PathVysion probe including: HER2 (red signal) and CEP17 (green signal) (**A1**); Poseidon™ Repeat Free™ including: TOP2A (17q21- green signal) & HER2 (17q12- red signal) & SE 17/CEP17 (light blue) Triple-Colour probe (**A2**); and the in-house probe kit with HER2 (red signal) and ACTG1 (green signal) (**A3**). These pictures exhibit case 15 that appeared to have centromeric amplification alone, along with chromosome 17 polysomy.

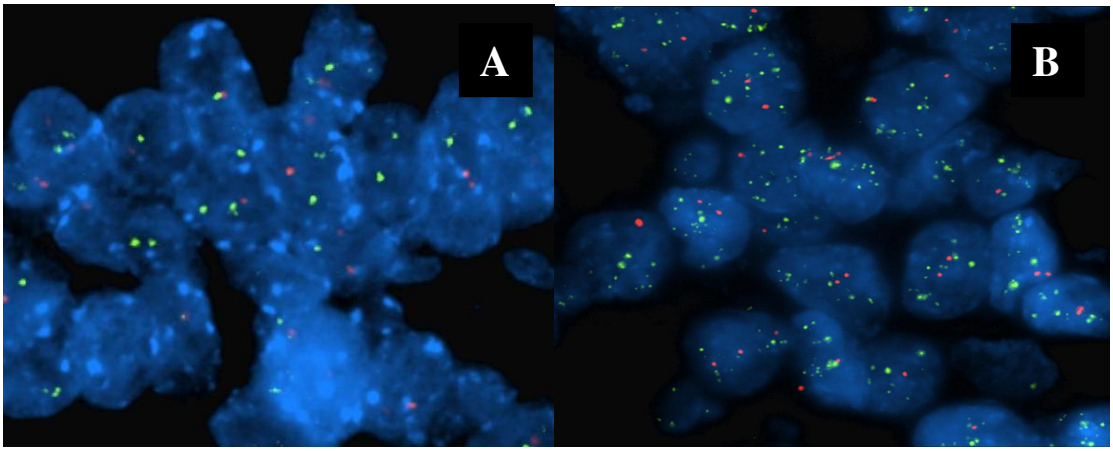

Figure 14: DAPI stained interphase nuclei hybridised with the Vysis LSI PML/RARA Dual Colour, Dual Fusion Translocation Probe kit. **A** shows case 17, which is negative for RARA amplification (green signals). **B** shows case 14, which is positive for RARA amplification. PML is represented by red signals, and RARA by green signals.

Table 6: Summary of the ratios obtained from the three probes for each ambiguous case.

Case	PathVysion HER2/CEP17 ratio	Poseidon HER2/CEP17 ratio	In-house HER2/ACTG1 ratio
1	1.3	1.2	11.7
2	2.8	1.9	16
3	1.3	1	6.5
4	1	1	17.3
5	1	1	13.6
6	1.3	1	10.3
7	1	1	9.1
8	1	1	8.4
9	1	2.2	5.5
10	1	1.8	10.8
11	1	1	5.6
12	1	1	10.8
13	1.6	1.42	0.81
14	1	1	6.9
15	0.85	0.15	2.3
16	0.33	1	1
17	2.6	4.2	11.9
18	1	1	8.5
19	0.1	0.1	1.1

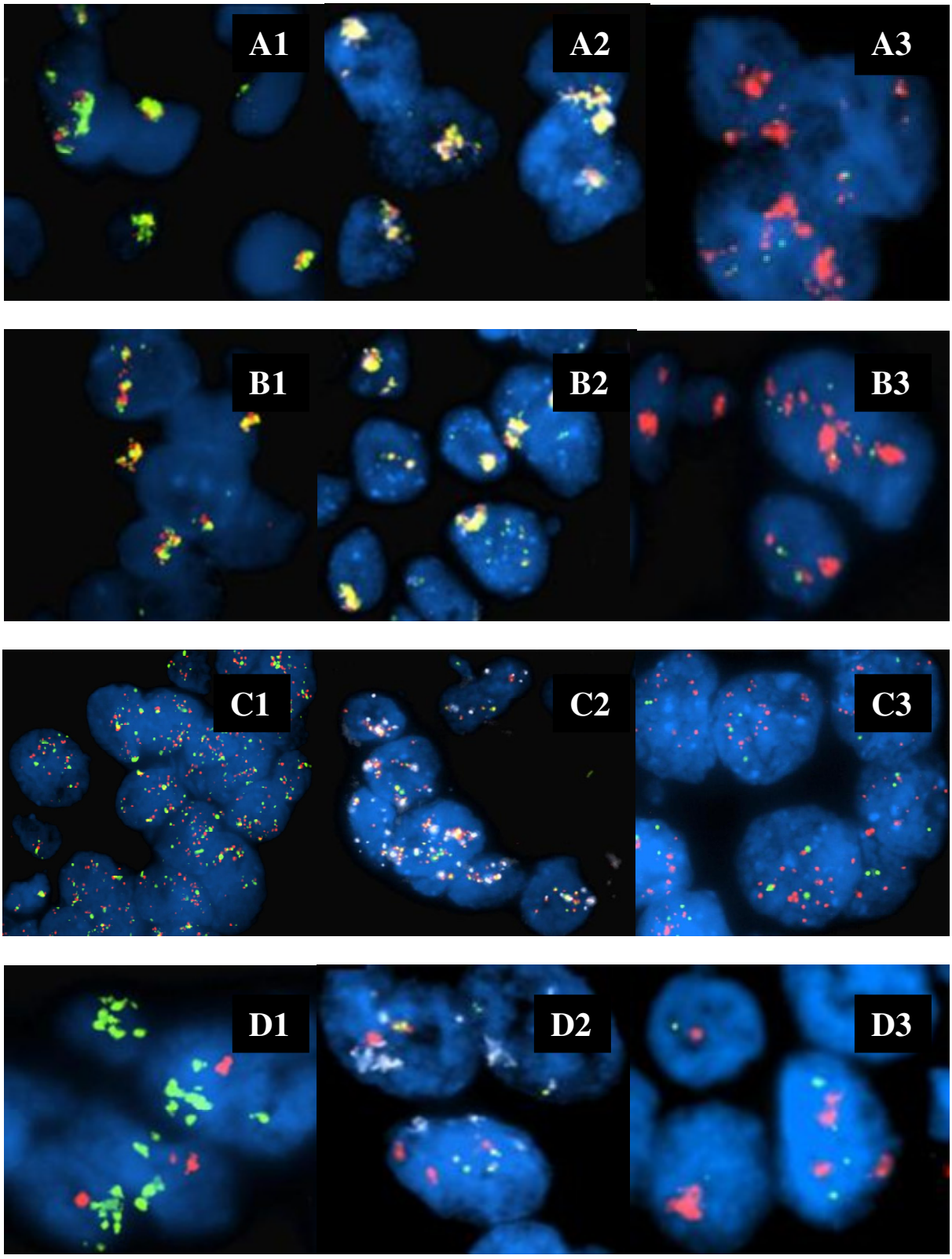

Figure 13 DAPI stained interphase nuclei of four ambiguous cases (**A,B,C,D**) hybridised with the Abbott-Vysis PathVysion probe (**A1**, **B1**, **C1**, **D1**; HER2 = red, CEP17= green); the Poseidon™ Repeat Free™ TOP2A (17q21) & Her2 (17q12) &

SE 17 Triple-Colour probe (**A2**, **B2**, **C2**, **D2**; HER2 = red, TOP2A = green, CEP17= light blue); and the in-house probe (**A3**, **B3**, **C3**, **D3**; HER2= red, ACTG1= green). The in-house kit shows the most unambiguous results with HER2 amplification clearly visible.

4.2.3. Validation of the in-house probe kit on previously established HER2 positive and negative samples

Based on the potential amplification of ACTG1 seen in the ambiguous case 13, The ACTG1/HER2 cocktail was tested on 20 HER2 positive and 20 HER2 negative samples (HER2 status was previously established during routines testing). This data could be used in determining if the in-house probe had the potential to be used commercially in routine HER2 testing. The results of the positive and negative samples tested with the in-house ACTG1/HER2 cocktail were compared to results seen for these samples in routine testing with the PathVysion probe (**Table 7**). Results from the in-house kit were mostly concordant with those of the PathVysion kit, however, ACTG1 amplification (defined as ≥ 6 ACTG1 copies) was seen in HER2 positive sample 13, and HER2 negative samples 25, 26 and 29 **(Figure 15).**

Table 7: Results of in-house probe tested on HER2 positive and negative samples

Positive samples

Sample name	PathVysion Common pattern (HER2 /CEP17)	HER/CEP17 ratio	Homemade probe Common Pattern (HER2/ACTG1)	HER2/ACTG1 ratio
1	>20r 2g	>2.2	>20r 2g	>2.2
2	>20r 2-4g	>2.2	>20r 1-2g	>2.2
3	>20r 2g	>2.2	>20r 1-2g	>2.2
4	>20r 1-2g	>2.2	>20r 1-3g	>2.2
5	>20r 2g	>2.2	>20r 0g	>2.2
6	>20r 2-3g	>2.2	>20r 2g	>2.2
7	>20r 2g	>2.2	>20r 1-2g	>2.2
8	>20r 2-3g	>2.2	>20r 2-4g	>2.2
9	>20r 2g	>2.2	>20r 2g	>2.2
10	>20r 2g	>2.2	>20r 3-6g	>2.2
11	>20r 2-4g	>2.2	>20r 2g	>2.2
12	>20r 1-2g	>2.2	>20r 2g	>2.2
13	>20r 2g	>2.2	>20r 3-6g (6-8g) (10%)	>2.2
14	>20r 2g	>2.2	>20r 2g	>2.2
15	>20r 1-2g	>2.2	>20r 2-4g	>2.2
16	>20r 2g	>2.2	>20r 1-2g	>2.2
17	>20r 2g	>2.2	>20r 2g	>2.2
18	>20r 2g	>2.2	>20r 2-3g	>2.2
19	>20r 2g	>2.2	>20r 2-3g	>2.2
20	>20r 2g	>2.2	>20r 2-4g	>2.2

Negative samples

Sample name	PathVysion Common pattern (HER2 /CEP17)	HER/CEP17 ratio	Homemade probe Common Pattern (HER2/ACTG1)	HER2/ACTG1 ratio
21	2r 2g	1	2r 2g	1
22	2r 2g	1	1-2r 1-2g	1
23	2r 2g	1	2r 2g	1
24	2r 2g	1	2r 2g	1
25	2r 2g	1	2-3r 2-3g	1
26	2r 2g	1	2r 3-7g	<1
27	2r 2g	1	2r 3-6g	<1
28	2r 2g	1	2r 2g	1
29	2r 2g	1	3r 3g (50%) 2r 2g (50%)	1
30	2r 2g	1	2r 2g	1
31	2r 2g	1	2r 2g	1
32	2r 2g	1	2r 2-3g	1
33	2r 2g	1	2r 2g	1
34	2r 2g	1	3-4r 3-4g	1
35	2r 2g	1	2-4r 2-3g	1.2
36	2r 2g	1	2r 2g	1
37	2r 2g	1	2r 2g	1
38	2r 2g	1	2r 2g	1
39	2r 2g	1	1-2r 4-6 g	<1
40	2r 2g	1	2r 2g	1

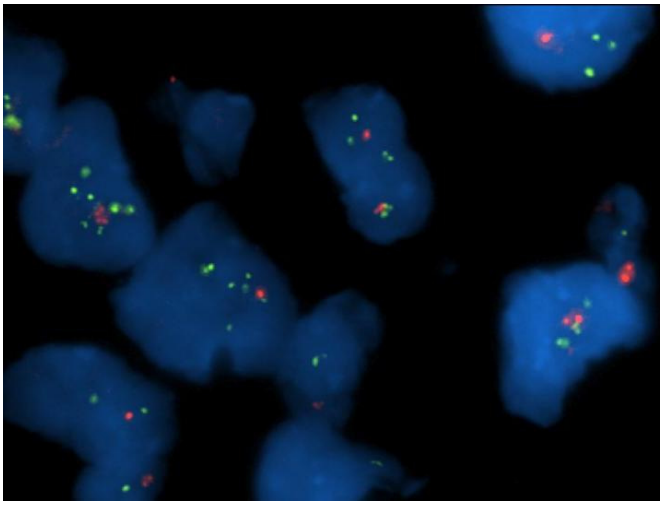

Figure 15: DAPI stained interphase nuclei of negative control hybridised with the in-house HER2/ACTG1 probe cocktail. The green signals represent increased ACTG1 copy number (ACTG1>6).

5. DISCUSSION AND CONCLUSION

5.1. Analysis of HER2 positivity in breast cancer samples submitted to our laboratory at Charlotte Maxeke Johannesburg Academic Hospital for routine FISH testing

In this study, data from the samples sent to our laboratory, were used to determine trends in the rate of HER2 amplification amongst South African patients. Samples are received from various parts of the country (for example, public hospitals in Cape Town, Port Elizabeth, Johannesburg, Pretoria, Kimberley, as well as a number of private laboratories that have branches across the country), and are therefore a good representation of South African breast cancer patients. The first observation was that the rate of HER2 amplification across patients in this cohort was much higher (44%) than the expected rate of HER2 amplification (the rate of HER2 amplification is 20-30% according to Slamon et al., 1987). The likely reason for this is that most samples are sent to our laboratory at the Charlotte Maxeke Johannesburg Academic Hospital for FISH testing subsequent to IHC testing. This means that there is a selection bias towards samples that are positive or equivocal for HER2 protein expression. The second observation is that the rate of HER2 amplification was significantly higher ($p < 0.05$) amongst African patients (52%) than amongst Caucasian patients (43%). This was not an expected result, as many studies from the US have shown that there is no statistical difference in the prevalence of HER2 amplification amongst African-American and European-American women (Amend et al., 2006; Stark et al., 2008; Stark et al., 2005; Al-Abbadi et al., 2006).

The data from the early onset breast cancer (≤ 35 years old) cohort showed three interesting trends. Firstly, in Caucasian women, the rate of the HER2 gene amplification in the younger group (68%) was significantly higher ($p< 0.05$) than in the general group of Caucasian patients (43%). This has been observed in other studies and the higher incidence of HER2 over-expression confers a more aggressive phenotype, which is typical of early onset breast cancer (Anders et al., 2008; Yankaskas, 2005; Hartley et al., 2006). Interestingly, the same was not seen amongst African women: the rate of the HER2 gene amplification in the younger group (49%)

was similar to that of the general African women's group (52%). Other studies that have reported increased HER2 gene amplification and protein expression in younger women did not stratify the patients by ethnicity, which may explain our differing results in the African cohort (Anders et al., 2008; Yankaskas, 2005; Hartley et al., 2006). Finally, the incidence of early onset breast cancer was significantly higher amongst African women than Caucasian women. This trend has been observed before by studies in the US. For example, analysis of the Surveillance, Epidemiology, and End Results (SEER) database has shown that breast cancer incidence rates are higher in African women compared to Caucasian women in the very young (less than 35) age group (Yankaskas, 2005).

Many studies have shown unequivocally that African women have larger and more aggressive tumours compared to Caucasian women (Parkin et al., 2008). This trend has also been observed in South Africa. For example, Vorobiof et al. (2001) showed that 77.7% of stages 3 and 4 breast cancer were found in African women, compared to 30.7% in Caucasian women, based on medical records from several provincial hospitals from 1970-1997 (Vorobiof et al., 2001). It was previously thought that this could be explained by African women delaying the seeking of medical attention as well as poorer medical care. However, studies in African-American women have shown that survival in African females is poorer than in Caucasian females even within the same categories of stage, and when adjusting for differences in health-care (Parkin et al., 2008). It is now widely suspected that the tumour biology of African women may be inherently more aggressive (Parkin et al., 2008). The future study of these African women with early onset breast cancer may give clues as to the genes playing a role in the increased aggressiveness of tumours amongst African women.

5.2. Investigation of ambiguous HER2 amplification cases by Fluorescent *in situ* Hybridisation with four different FISH probes

A major aim of this study was to show that CEP17 was amplified in ambiguous cases, making the routine analysis of these cases both challenging and potentially misleading. Ambiguous cases presented with clusters of innumerable red and green signals; or green signals too numerous to be accounted for by chromosome 17 polysomy (> 6 CEP17 signals). For the purpose of this study, which was to show centromeric amplification unequivocally, we selected 19 cases with more than 9 copies of CEP17 signals. Of these 19 ambiguous cases, 16 had centromeric amplification, as determined by amplified CEP 17 signals seen in both the PathVysion and Poseidon probes. Of the remaining three cases, one (case 17) only had high polysomy (4-6 CEP 17 signals with Poseidon probe) or potential artefact (5-10 CEP17 signals with PathVysion probe) with HER2 amplification. One (case 16), that had what appeared to be centromeric amplification, was in fact artefact/background. The last one (case 13) appeared to be polysomic (2-4 CEP17 signals with Poseidon probe) without HER2 amplification, but with increased ACTG1 copy numbers.

Cases with chromosome 17 centromeric amplification have been seen elsewhere. In a study by Troxell et al. (2006), using SMS (Smith-Magenis syndrome critical region-17p11.2) and RARA (Retinoic acid receptor-17q21.2) as surrogate FISH controls, five of seven investigated cases were found to have centromeric amplification (Troxell et al., 2006). Two had centromeric amplification alone; two had amplicons that encompassed HER2 and CEP17, and one that encompassed HER2, CEP17 and RARA. In a recent study by Marchio et al. (2009), using microarray-based comparative genomic hybridization, as well as SMS and RARA probes, of 18 supposedly polysomic cases, 5 were found to have amplification of CEP17, 11 had 17q gains involving CEP17, 1 was true polysomy and the other showed 17q gain without CEP17 involvement (Marchio et al., 2009). As was seen by Troxell et al. (2006) and Marchio et al. (2009), we also observed two cases (case 15 and case 19) that had centromeric amplification alone (**Figure 12)** (Troxell et al., 2006; Marchio et al., 2009). This demonstrates that CEP 17 may not only be co-amplified with HER2 but may also, be it rare, be subject to its own rearrangements in breast cancer. These

two studies, in conjunction with the high level of centromeric amplification seen here, strongly support the use of a control other than CEP17 in HER2 testing. Although centromeric amplification only occurs at a frequency of 1.2%, accurate HER2 assessment is essential in each and every patient.

Studies have shown the need for an internal control probe in HER2 testing that is on chromosome 17, but not included in the HER2 amplicon (Sauter et al., 2009; Dal Lago et al., 2006). Presently, all commercially-available dual-probe kits use CEP17 as an internal control, which this study has shown to be inadequate. In their study, Troxell et al. (2006) proposed the use of SMS and RARA as alternative controls in the routine testing of ambiguous HER2 cases (Troxell et al., 2006). It is clear from this study and that of Marchio et al. (2009) that RARA is a common component of the HER2 amplicon, and the results of Marchio et al. (2009) showed the use of SMS as a control probe to be limiting (Marchio et al., 2009). Therefore, the second aim of this study was to develop a FISH probe that would act as a superior control to CEP17 in HER2 testing. ACTG1, located on the long arm of chromosome 17, was chosen as a potential alternative because it is a well conserved house-keeping gene, it is located 416 megabases from HER2, and there was no evidence of it playing a role in breast cancer, according to a search of all publicly available data. The ACTG1 gene proved to be a very good control in ambiguous cases. The images clearly showed how HER2 amplification could be seen clearly and unequivocally in ambiguous cases when this probe was used as a control (**Figure 13**). Additionally, the HER2/ACTG1 ratios were significantly higher than those of HER2/CEP17, seen in both the PathVysion and Poseidon probe kits. Most of the ambiguous cases with centromeric amplification had HER2/CEP17 ratios that would result in them being classified as negative (according to international guidelines), yet became positive (ratio >2.2) when assessed using the HER2/ACTG1 ratio.

Despite ACTG1 being a superior control in most ambiguous cases, in one ambiguous case, 13, the number of the ACTG1 gene signals was higher than those of the HER2 gene, which was not amplified. This was not an expected result, and suggested that there may be amplification occurring in this region. The in-house kit was subsequently tested on twenty previously established HER2 positive and twenty previously established negative samples, in order to determine the frequency of this

amplification and to assess if the ACTG1 gene had the potential to be used as an alternative control for HER2 testing in the clinical setting. While ACTG1 was reliable in most of the non-ambiguous sample group (36/40 samples), four samples, 13 (positive for HER2), 26, 27 and 39 (negative for HER2) also showed increased ACTG1 signals (3-8 signals), as was seen in the ambiguous case 13 (**Figure 15)**. Several studies have shown amplification on the long arm of chromosome 17 and on 17q25.3 in particular, which harbours potential driver genes such as the SYNGR2, PGS1, PSCD1 and BIRC5 genes, all of which have been shown to be amplified and over-expressed in Breast Cancer (Arriola et al., 2008; Hicks et al. 2009; Hwang et al., 2008, Bergamaschi et al., 2006; Fridyland et al., 2006). However, in the copy number alteration papers reviewed for this study, none have defined an amplicon on 17q25.3 that extends as far as ACTG1 (Arriola et al., 2008; Hicks et al. 2009; Hwang et al., 2008; Bergamaschi et al., 2006.; Fridyland et al., 2006). Additionally, the BIRC5 gene (the closest potential driver gene to ACTG1 gene in the proposed 17q25.3 amplicon) and ACTG1 gene are separated by approximately 3.2 megabases (BIRC5 location: 76,210,277-76,221,715; ACTG1 location: 79,476,999-79,479,827 according to Ensembl), which is a fair distance, making it unlikely that ACTG1 would be co-amplified along with BIRC5. Although increased ACTG1 copy number was limited ($\leq$ 8), there is substantial evidence showing a high level of amplification and alteration occurring on 17q (Pollack et al., 2002). Therefore, further testing to assess the value of this gene as a control is warranted.

Interestingly, it would appear from the preliminary results of this study that ACTG1 is more likely to be amplified in HER2 negative cases (four out of five samples with ACTG1 amplification were HER2 negative). This suggests the possibility of a driver gene in this region that may potentially be driving carcinogenesis in HER2 negative breast cancers. Further study of this region may yield a potential target for therapy.

Another aim of the study was to further elucidate the structure and components of the HER2 amplicon. This was done by assessing the TOP2A and RARA genes status in these ambiguous cases. The nature of RARA and TOP2A gene amplification in relation to HER2 was the same as documented in previous studies. For example, TOP2A was only amplified in HER2 positive cases. While it has been shown to be independently amplified (Nielsen et al., 2008), most studies see TOP2A amplification

in HER2 positive cases only (Jarvinen et al., 1999; Olsen et al., 2004; Hicks et al, 2005; Bhargava et al., 2005; Arriola et al., 2008). In this sample set, TOP2A mostly appeared to have the same copy number as HER2, but due to the high density of signals, it was often hard to count the exact number of green and red signals seen in the Poseidon probe. One case, 18, had differing HER2 and TOP2A gene copy numbers, yet both genes were clearly amplified. This supports other data suggesting that TOP2A and HER2 may be amplified in adjacent amplicons (Jarvinen et al., 1999; Olsen et al., 2004; Hicks et al, 2005; Bhargava et al., 2005). However, according to Jacobson et al. (2004) differing HER2 and TOP2A gene copy number can be explained by the BFB cycle process (section 1.3), assuming multiple potential break sites, differing genes copy number is not necessarily evidence for separate amplicons (**Figure 16)** (Jacobson et al., 2004). This explains the results seen in this study whereby most samples had HER2 and TOP2A genes in the same copy number, while case 18 had over 20 HER2 signals and 4-12 TOP2A signals. TOP2A gene deletion is a common feature in HER2 gene amplified cases (Nielsen et al., 2008; Hick et al., 2005), yet no TOP2A gene deletion was seen in any of the ambiguous cases studied here. It is, however, possible, that cases with TOP2A hybridisation that looked uninformative (i.e. 0-1 green TOP2A signals; ambiguous cases 1 and 4), had deletion of this gene. This would need to be confirmed by testing these cases with another FISH kit containing a TOP2A probe.

The RARA gene appeared to only amplify with TOP2A suggesting either a common amplicon as seen by Arriola et al. (2008) or possibly co-amplification of two separate amplicons (Arriola et al., 2008). RARA gene amplification in HER2 positive cases has implications regarding the use of ATRA in the treatment of breast cancer. ATRA won't be effective unless the RARA gene is amplified, but if HER2 is also amplified, these cells will become resistant to ATRA (Tari et al., 2002). Once treated with Herceptin, which reduces this resistance, ATRA may become an efficient additional therapy in the treatment of breast cancer.

5.3. Limitations and further research

The significantly higher percentage of HER2 positivity seen in African patients compared to Caucasian patients was not an unexpected result and showed a trend that has not been reported elsewhere. It is not possible to comment further within the scope of this study as it is not an epidemiological study. It is, however, likely to reflect a trend that requires further exploration. Also, despite there being a higher incidence of early onset breast cancer in African women, the data was not corrected for the differing age distributions of the two populations. Had this been done, results may have been different.

A further limitation of the study is that only cases with more than 9 CEP17 signals were included and investigated with the four FISH probe kits. The rationale was to show CEP 17 amplification unequivocally. However, at this stage, ambiguous cases with 6-9 copies of CEP17 can not yet be defined as either polysomy or amplification of the CEP17 region. Further studies would have to be done to assess these cases with an alternative control to CEP17. This may allow for further elucidation of the true definition of polysomy in HER2 testing.

Another major limitation of the study was that the ACTG1 gene, while extremely effective on ambiguous cases, was shown to be amplified in some of the control non-ambiguous samples. While this gene could replace CEP17 as an internal control in HER2 testing, cases with ACTG1 amplification would result in HER2/ACTG1 ratios below 1, which would be challenging when reporting FISH results in accordance with the ASCO/CAP guidelines. Future studies could further investigate the efficacy of ACTG1 as an alternative control. If found to be inadequate, further research opportunities would be to search for a gene on chromosome 17 that does not display any amplification. As seen in the literature review, many genes on chromosome 17 are gained or lost in breast cancer. Therefore, the employment of microarray technology on ambiguous cases may allow for the identification of an alternative internal control gene on chromosome 17 that could be implemented into HER2 FISH testing.

Another research opportunity that has arisen from this study is the exploration of the regions of amplification around ACTG1. As shown here, the region is more likely to

be amplified in HER2 negative cases and there is a possibility of a driver gene in this region that may be playing a role in breast tumours without HER2 amplification.

5.4. Conclusion

In conclusion, this study has clearly shown the amplification of chromosome 17 centromere in ambiguous cases, which strongly supports the need for an alternative control probe in routine HER2 testing. While the ACTG1 gene proved to be a very effective control in ambiguous cases, further testing on positive and negative samples showed it may not be the best alternative. However, amplification of the ACTG1 gene in mostly HER2-negative samples suggests a possible driver gene nearby that could potentially be identified as therapeutic target in the future. The TOP2A gene was only amplified in HER2 positive cases. The differing copy number of these two genes in one case can be explained by alternating break points in BFB cycles. The RARA gene was only amplified when the TOP2A gene was also amplified, and this in conjunction with their close proximity suggests that they are can either be amplified together as a unit, or in conjunction with HER2.

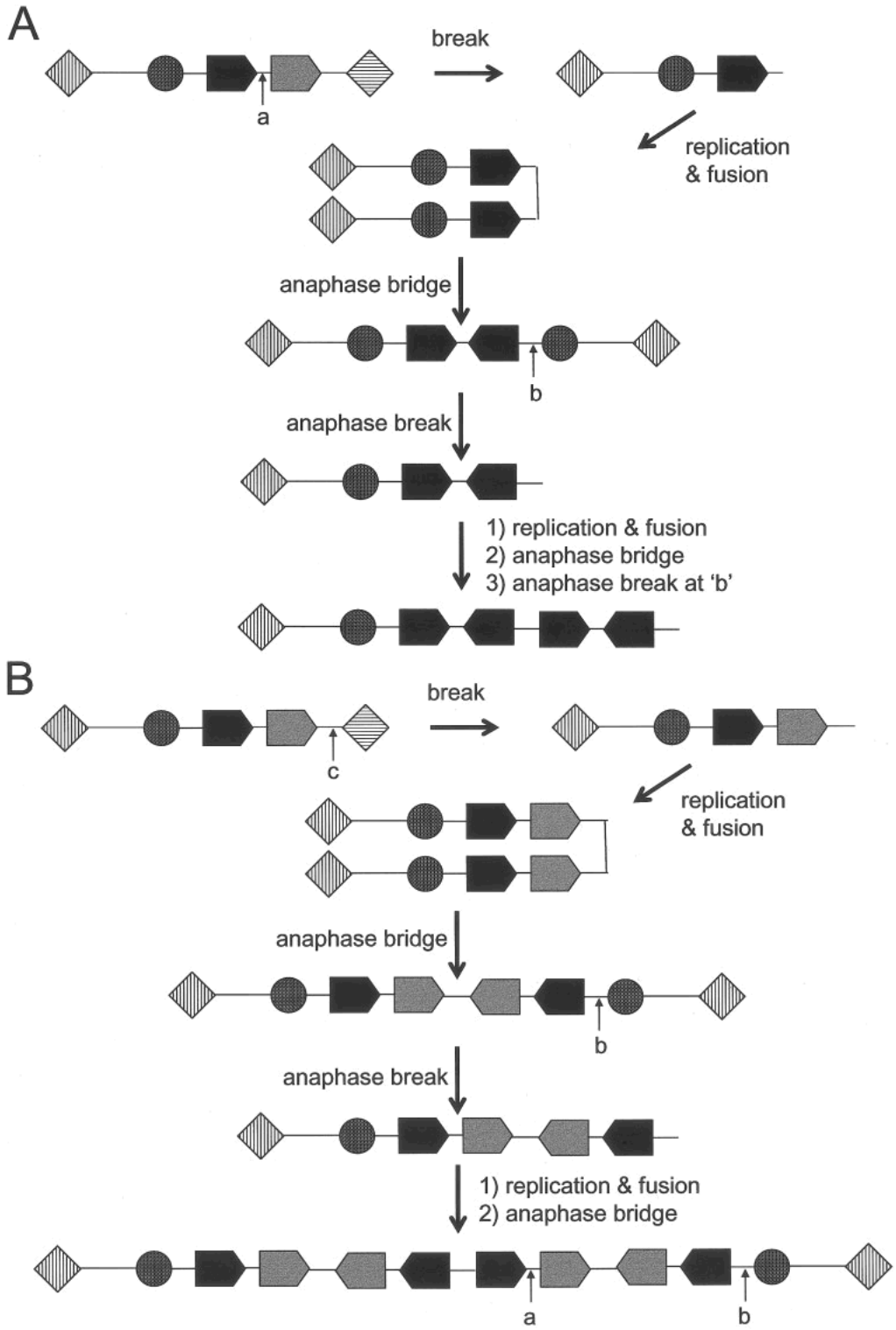

Figure 16: **A** shows HER2 amplification. **B** shows amplification of HER2 and TOP2A. Diamonds represent telomeres, circles represent centromeres. The pentagons represent HER2 (black) and TOP2A (grey). a, b and c represent breakpoints. Breaks in the order of **c** and **b** alternately would result in HER2 and TOP2A genes being co-amplified with the same copy number. However, if breaks occurred in the order of **c**, **b** and **a,** the two genes will have differing copy numbers.

6. APPENDIX A:

LB broth

10g Bacto tryptone (Merck®)

5g Bacto-yeast extract (Merck®)

5g NaCl (Merck®)

Make to 1 litre

Autoclave

Glycerol Solution

65% glycerol (Merck®)

0.1M $MgS0_4$ (Merck®)

0.025 M Tris-HCl pH8.0 (Merck®)

Agar Plates

7.5 Agar (Difco®) in 500ml LB broth

Autoclave

Add 20ug/ml chloramphenicol (Sigma®)

Pour 25ml into Petri dishes and allow to set.

2% Agarose gel

2g of Agarose powder (BioLine) dissolved in 100 ml 1X TAE buffer

3 µl Ethidium Bromide or 10 µl Gel Red

Heat until clear

Pours 3 gels

Dnase 1 Stock

3mg DNase1 (Boehringer Mannheim)

0.5ml of 0.3M NaCl (Analysed Analytical Reagents)

0.5ml of glycerol solution

made up to 1ml with distilled water

stored at -20°C

10 X Nick Translation buffer:

0.5M Tris (Sigma®)

50mM $MgCl_2$ (Sigma Aldrich®)

0.5mg/ml Bovine Serum Albumin (Roche®)

0.1M β-mercaptoethanol

0.1ml β-mercaptoethanol (Sigma®)

14.4ml of double distilled water

dNTP (Promega) mix

Reagent	Volume(µl)
dATP(10mM)	1.5
dCTP(10mM)	1.5
dGTP(10mM)	1.5
dTTP(10mM)	0.75
Spectrum Orange/Green(1mM)	7.5
Distilled water	17.25
Total	30

Hybridisation buffer

50% deionised formamide (Merck®)

2 x SSC

10% dextran sulphate (Sigma®)

50 mM sodium dihydrogen orthophosphate (Merck®)

pH to 7.0

Store at -20°C

20X Saline Sodium Citrate (SSC)

3M NaCl (Analysed Analytical Reagent)

0.3M sodium citrate (Merck®)

pH to 7

1 in 10 dilution of above makes 2X SSC

1M Sodium Thiocyanate (NASCN)

81.08g NASCN (Sigma Aldrich®) in 1L water

1% Formaldehyde:

27ml 37% Formaldehyde (Saarchem®) in 900ml H_2O

pH to 7

Complete Medium

50 ml foetal bovine serum (Sigma®)

5 ml streptomycin (5000 µg/ml, Highveld Biological)

445 ml RPMI 1640 medium

Denaturing solution

35ml Deionised Formamide (Merck®)

5ml 20X SSC

5ml Phosphate Buffer (recipe below)

5ml dH2O

pH to 7.0

Phosphate buffer (pH7)

Solution A: 4.54g of KH_2PO_4 per 500ml

Solution B: 5.95g of $Na_2PO_4.2H_2O$ per 500ml

In 100ml solution add: 41.3ml of solution A

58.7ml of Solution B

pH to 7 using either solution A(acid) or solution B(base).

DAPI stock

10mg of DAPI Stock (Merck®) in 50ml of 2xSSC

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