Metasin: An Intra-Operative Real-Time Quantitative Reverse Transcription Polymerase-Chain Reaction (RT-qPCR) Assay to Detect Metastatic Breast Cancer in Sentinel Lymph Nodes

Dr Salma Al-Ramadhani

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To my parents, Dr Saad Al-Ramadhani and Mrs Nahla Hasan, nothing would have been possible without your unwavering financial support, help, advice, guidance and love. I am forever indebted to you both.

To my twin brother Omar, your continuous encouragement and support during my MD made the impossible task seem possible. You have always supported me in my life and I cannot thank you enough.

ABSTRACT

The most important prognostic factor in breast cancer is the presence or absence of metastases in axillary lymph nodes. Frozen section and touch imprint cytology are conventional intra-operative methods used in the detection of metastatic breast cancer with varying sensitivities and specificities. The limitation of these methods led to the development of alternative molecular diagnostic tests, such as GeneSearch, a commercial real-time quantitative Polymerase Chain Reaction (RT-qPCR) assay that allows for an intra-operative diagnosis of metastatic breast cancer. When the GeneSearch assay was discontinued, Metasin was developed as an in-house RT-qPCR replacement assay. Metasin targets the epithelial cell marker cytokeratin 19 (CK19) and the breast marker mammaglobin (MGB) mRNA to confirm the presence or absence of metastatic disease, whilst the reference gene porphobilinogen deaminase (PBGD) acts as a positive control for the performance of the assay. The optimised assay can produce a result within 32 minutes allowing it to be used in the intra-operative setting to detect metastatic breast cancer in sentinel lymph nodes.

154 archived lymph node homogenates that were previously analysed by both GeneSearch and histology in parallel were used to validate Metasin. Out of 154 cases, 148 showed concordance with both GeneSearch and Metasin with 111 cases being negative and 37 cases being positive. There were six discordant cases, four in which only Metasin detected metastases and two in which only GeneSearch picked up metastases. Out of the four Metasin-only positive cases, three were found to be positive on histology after deeper levels were cut in the slices sent for histological assessment. Therefore, one case could not be shown histologically to be positive for metastases. There were two cases that were missed by Metasin but picked up by GeneSearch. One case was positive on histology and the second case negative for histology. The error rate for Metasin was 3.89%. The sensitivity and specificity of the Metasin assay were found to be 95% and 98% respectively, and the positive and negative predictive values were 90% and 98% respectively. These results are comparable to those of GeneSearch. Metasin had an assay time of less than 45 minutes and was operated by biomedical scientists. The results of the validation

process were deemed acceptable for the assay to be run live and used in the clinical setting. Metasin continues to provide breast cancer patients at Princess Alexandra Hospital with all the advantages that a molecular intra-operative diagnostic service provides.

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ABBREVIATIONS

AC:	Axillary Clearance
ALND:	Axillary lymph node dissection
cDNA:	Complementary Deoxyribonucleic Acid
Cq:	Quantification Cycle
Ct:	Cycle Threshold
CK19:	Cytokeratin 19
DCIS:	Ductal Carcinoma in situ
ddNTPs:	Dideoxynucleoside Triphosphates
DNA:	Deoxyribonucleic Acid
dNTPs:	Deoxyribonucleotide Triphosphates
dsDNA:	Double Stranded Deoxyribonucleic Acid
EGFR:	Epidermal Growth Factor Receptor
ER:	Oestrogen Receptor
EST:	Expressed Sequence Tag
FDA:	Food and Drug Administration
FNAC:	Fine Needle Aspiration Cytology
FS:	Frozen Section
GAPDH:	Glyceraldehyde-3-Phosphate Dehydrogenase
GGI:	Gene Expression Grade Index
GITC:	Guanidine Isothyocyanate
H&E:	Haematoxylin and Eosin
Her-2:	Human Epidermal Growth Factor 2
HNPCC:	Hereditary Nonpolyposis Colon Cancer
HOXB13:	Homeobox Gene 13
IDC, NOS:	Invasive Ductal Carcinoma, Not Otherwise Specified
IHC:	Immunohistochemistry
IL17BR:	Interleukin 17B Receptor
ILC:	Invasive Lobular Carcinoma
ITCs:	Isolated Tumour Cells
MDT:	Multidisciplinary Team

MGB:	Mammaglobin
MLH1:	MutL Homologue 1
mm:	Millimetre
MPSS:	Massively Parallel Signature Sequencing
MRI:	Magnetic Resonance Imaging
mRNA:	Messenger Ribonucleic Acid
miRNA:	Micro Ribonucleic Acid
MSH2:	MutS Homolgue 2
NICE:	National Institute for Health and Care Excellence
NPV:	Negative Predictive Value
PBGD:	Porphobilinogen Deaminase
PCR:	Polymerase Chain Reaction
PPV:	Positive Predictive Value
PR:	Progesterone Receptor
QOL:	Quality of Life
RCI:	Rapid Cytokeratin Immunostaining
RISC:	Ribonucleic Acid Induced Silencing Complex
RNA:	Ribonucleic Acid
RS:	Recurrence Score
RT:	Reverse Transcription
RT-PCR:	Reverse Transcription Polymerase Chain Reaction
RT-qPCR:	Real Time Quantitative Reverse Transcription Polymerase Chain Reaction
s:	Second
SAGE:	Serial Analysis of Gene Expression
SCGB2A2:	Secretoglobin Family 2A, Member 1
siRNA:	Short Interfering Ribonucleic Acid
SLN:	Sentinel Lymph Node
SLNB:	Sentinel Lymph Node Biopsy
SOP:	Standard Operating Procedure
TIC:	Touch Imprint Cytology
Tm:	Melting Temperature

UPL: Universal Probe Library

US: Ultrasound

UV: Ultraviolet

VACB: Vacuum Assisted Biopsy

PART I. INTRODUCTION

The introduction gives a brief overview of the characteristics, diagnosis and management of breast cancer and particularly focuses on detecting lymph node metastases and the concept of the sentinel lymph node. It then goes on to describe the conventional methods used in intra-operative diagnosis of metastases in sentinel lymph nodes. Molecular pathology and its application in medicine and in particular breast cancer is briefly introduced with emphasis on real-time quantitative polymerase chain reaction (RT-qPCR) and its application in the GeneSearch assay. Finally, an analysis of GeneSearch from various studies is discussed and the aims of the investigation expanded.

Chapter 1

1.1   Breast cancer

This chapter aims to describe the epidemiological and histopathological characteristics of breast cancer, including the main histological subtypes and the criteria required for grading and staging the cancer. The diagnosis and management of patients is discussed with a more detailed description of the concept of sentinel lymph nodes and the current intra-operative diagnostic techniques of frozen section and touch imprint cytology to detect metastatic tumour. Molecular pathology is introduced as an important emerging tool used in the diagnosis and management of patients with breast cancer with emphasis on gene expression profiling and the role of RT-qPCR. A brief technical description of how PCR works is also provided. Finally, the concept of the GeneSearch assay and a review of its performance is described.

1.1.1   Epidemiology of breast cancer

Breast cancer is the commonest cancer in women in the UK accounting for 31% of all new cancer cases in women. The latest figures show that there were 48,788 new cases of breast cancer (371, <1% in men) in 2009. The incidence of breast cancer in women is

strongly related to age. 81% of cancers were diagnosed in the over 50 age group. 48% of breast cancer cases are diagnosed in the 50-69 year old age group where breast screening is offered (CRUK, 2012).
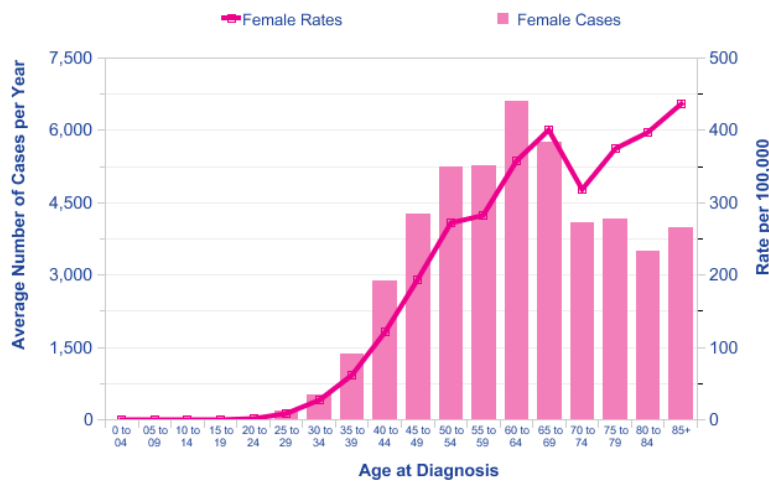
Figure 1: Distribution of breast cancer according to age (CRUK, 2012)

1.1.2 Risk factors for breast cancer

Risk factors for breast cancer include the following:

- Female gender
- Peak incidence between the ages of 75-80 years
- Early age of menarche (women who had their menarche younger than 11 have a 20% increased risk of breast cancer)
- Nulliparous women or women over 35 years having a full term pregnancy have a higher risk than women less than 20 years having a full term pregnancy. Breastfeeding is thought to lower the risk of breast cancer.
- A family history of first degree relatives with breast cancer
- Non-hispanic white women have the highest rates of breast cancer. 1 in 15 in this group develop invasive cancer within 20 years after age 50

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- Exposure to oestrogen, such as in women who take hormone replacement therapy (HRT), is a risk factor for developing ER-positive breast cancer
- BRCA1 and BRCA2 are susceptibility genes in which a single mutation can lead to breast cancer. These mutations account for 3 – 5% of all breast cancers
- A higher body mass index in postmenopausal women (Tavassoli and Devilee et al, 2003)

1.1.3 Histological subtypes of breast cancer

The World Health Organisation classification of breast tumours lists many different types of epithelial tumours, all with their own histopathological appearances and prognosis (Tavassoli and Devilee et al, 2003). Typing invasive carcinomas is known to have prognostic value and also provides information on the pattern of metastatic spread and tumour behaviour (Ellis et al, 2005). The two commonest types of invasive breast cancer are infiltrating ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) (Tavassoli and Devilee et al, 2003).

Infiltrating Ductal Carcinoma

Infiltrating ductal carcinoma, not otherwise specified (NOS) is the commonest type of breast cancer accounting for 41-75% of all mammary invasive carcinomas in seven published series (Elston and Ellis, 2000) and is a diagnosis of exclusion. Therefore, it is a tumour that cannot be classified as any special type ie Not Otherwise Specified, (NOS). The histological appearances vary considerably with tumour architecture ranging from solid to glandular, cords and single cells. The neoplastic cells also vary in appearance ranging from mildly atypical to highly pleomorphic (Tavassoli and Devilee et al, 2003).
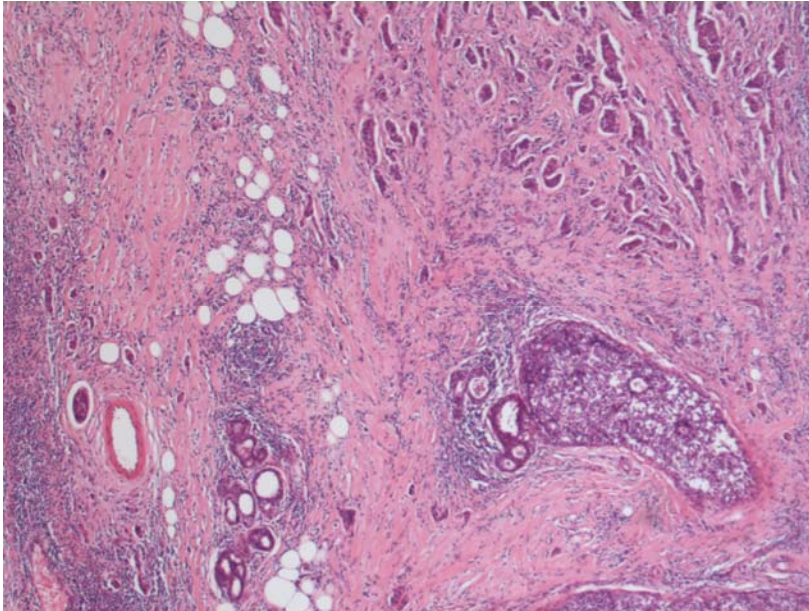
Figure 2. Invasive ductal carcinoma in a desmoplastic stroma. Lymphovascular invasion is seen on the left. (x20)

Invasive Lobular Carcinoma

This tumour can be difficult to detect, with bland single tumour cells infiltrating the breast tissue. Classically the carcinoma cells are lined up in single file pattern. There is targetoid growth with a circumferential growth pattern around ducts and lobules. The neoplastic cells can be difficult to detect as the nuclei can look mildly atypical with slight hyperchromasia. Intracytoplasmic inclusions and signet-ring cells can be seen. Immunohistochemistry (IHC) can be done for e-cadherin, a cell adhesion molecule, the absence of which would indicate ILC. Metastases to axillary lymph nodes can be very difficult to detect due to the non-descript and subtle appearance of the tumour cells (Tavassoli and Devilee et al, 2003).
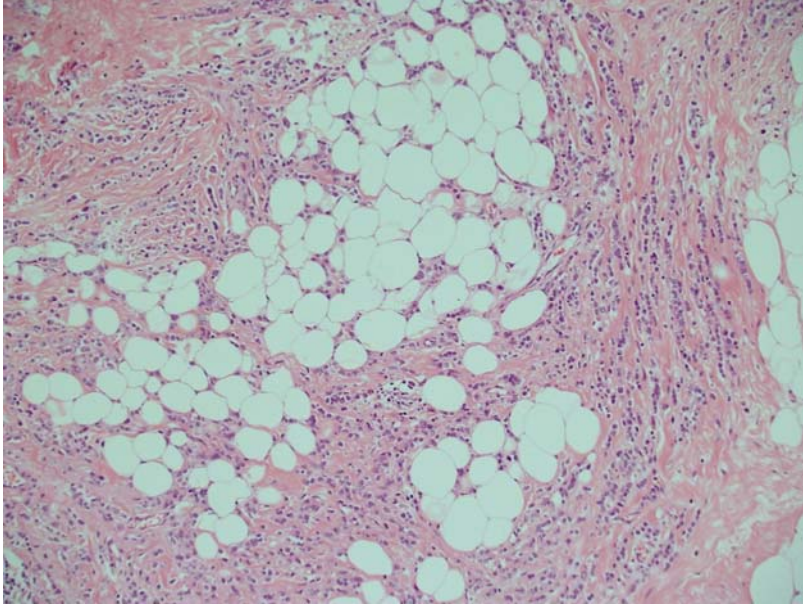
Figure 3 Invasive lobular carcinoma showing characteristic features of the malignant cells arranged in single file and singly. (x20)

1.1.4 Grading breast cancer

Grading a cancer is the assessment of its degree of differentiation and in some cancers, the number of mitoses and architectural features (Kumar et al, 2010). Grading of breast cancer is carried out using the Nottingham/Elston and Ellis grading system (Ellis et al, 2005). It involves assessing three parameters, each of which has a score of 1 - 3:

1. Tubule, glandular or acinar formation: the greater the percentage of tubules, the lower the score. If more than 75% of the cancer consists of tubules, it scores 1, between 10 and 75%, scores 2 and less than 10%, a score of 3.

2. Nuclear pleomorphism: slightly enlarged, regular nuclei score 1, moderately pleomorphic nuclei score 2 and highly pleomorphic nuclei score 3

3. Mitotic count: the least differentiated area of the tumour is scanned and the number of mitoses in 10 high power fields counted. Depending on the field

diameter, a table listing the number of mitotic figures corresponding to a score
is read.

The total score of all three parameters is added up and for those scoring 3-5 the
tumour is a grade 1, a score of 6-7 a grade 2 and a score of 8-9 a grade 3, the worst
grade.

There is a highly significant relationship between histological grade and prognosis
whereby the overall survival and recurrence-free interval are worse in patients with
grade 3 or poorly differentiated tumour than those with grade 1 or well differentiated
tumours. This is illustrated in the graph below (Elston and Ellis, 2000).

399	356	179	76 Grade 1
686	529	241	100 Grade 2
920	520	219	95 Grade 3
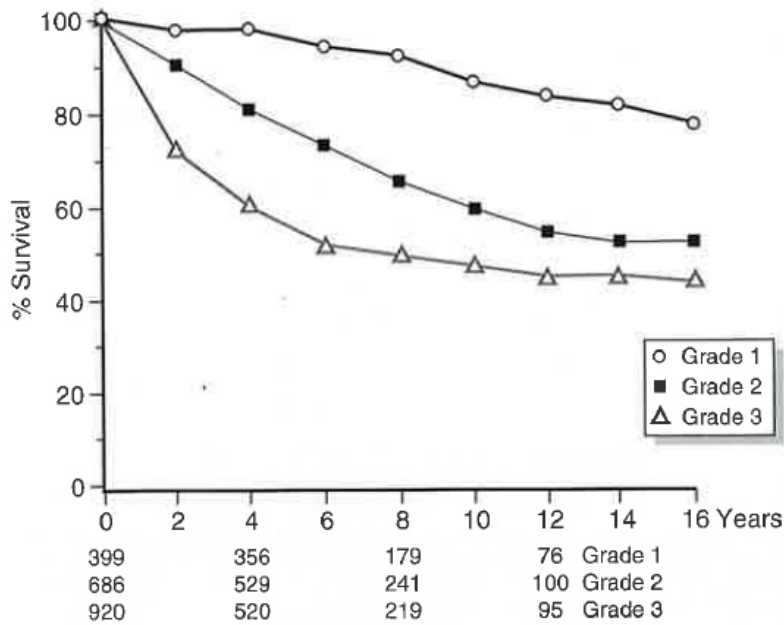
Figure 4. The graph illustrates the correlation between histological grade and overall
survival in women with primary operable breast cancer. The overall survival of women
with grade 1 breast cancer is better than those with grade 2 or grade 3 breast cancer
(Elston and Ellis, 2000).

1.1.5   Staging breast cancer

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Staging a cancer is a combined clinical and pathological assessment. It is based on the extent of lymph node spread and presence or absence of metastases. The system used to stage breast cancer is the TNM system where T is the size of the tumour and the extent of spread ie to the skin or chest wall, N is the number of lymph nodes affected and M is the presence or absence of distant metastatic spread (Ellis et al, 2005).

1.1.6 Important prognostic information

The important points to document when reporting breast cancers are the ones that hold prognostic information. Poor prognostic indicators include certain tumour subtypes, large tumour size, high tumour grade, presence of lymphovascular invasion and presence of metastatic spread to the lymph nodes (Ellis et al, 2005). This last feature is one of the most important prognostic indicators.

1.1.7 Metastases, their mode of spread and Isolated Tumour Cells (ITCs)

Tumour implants at a distant site from the primary tumour are metastases. This characteristic defines a tumour as being malignant as benign tumours are not known to metastasize. Around 30% of patients newly diagnosed with a malignant tumour present with metastases and 10% of breast patients have metastasis at the time of presentation (Kumar et al, 2010). The larger, more aggressive, faster growing tumours are the most likely to spread, however, there are exceptions to this rule.
The commonest sites of metastatic spread in breast cancer are the bone, lungs and liver (Kumar et al, 2010).
There are three ways tumours can spread to other organs of the body:

- Direct seeding of cavities or surfaces: this is when a tumour penetrates into a cavity, such as peritoneal, pericardial and pleural cavities. They can then spread directly to other organs within the space.
- Haematogeneous spread: tumour cells can infiltrate veins and less commonly arteries. Tumour cells invading into venous blood vessels follow the venous flow, resting in the first capillary bed they meet. The liver, which receives

portal blood, and the lungs, which receive caval blood, are the two commonest sites for metastasis (Kumar et al, 2010).

- Lymphatic spread: this is the commonest way in which carcinomas, such as breast cancer, spreads. The tumour infiltrates the functional lymphatics close to the tumour edge and follows the natural route of lymphatic drainage. Carcinomas of the breast that arise in the upper outer quadrant normally metastasize to the axillary lymph nodes and those located in the inner quadrants spread to the nodes along the internal mammary nodes. The infraclavicular and supraclavicular lymph nodes are usually the last group to be affected and signify the extensive spread of the tumour. The tumour can then spread from the nodes through the lymphatics to distant sites in the body (Kumar et al, 2010).
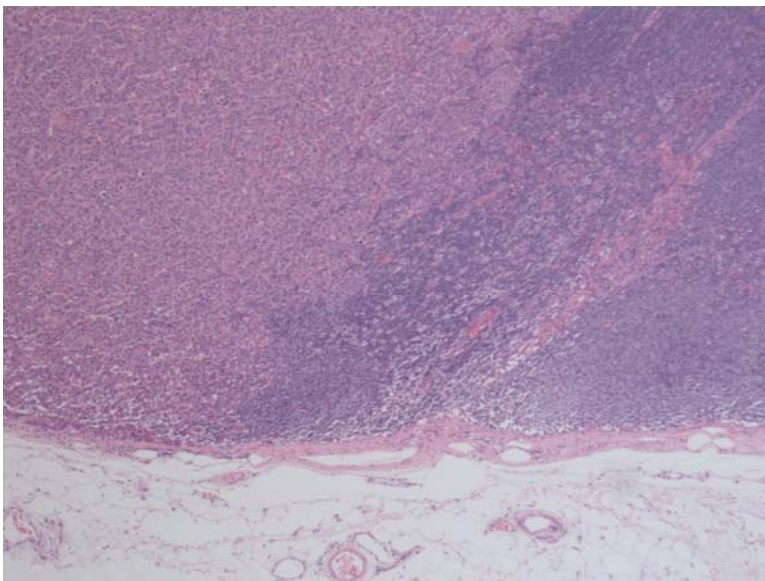
Figure 5. A lymph node showing metastatic IDC on the left and normal lymphoid tissue on the right. (x20)

Breast cancer metastases to the lymph node are classified as either macrometastases, micrometastases or isolated tumour cells based on the size of the deposit (Ellis et al,

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2005). The larger the size of the deposit, the greater the number of metastatic cells there are. Macrometastases are considered to be greater than 2mm. Micrometastases are one or more deposits of tumour measuring between 0.2mm up to 2mm. Isolated tumour cells (ITCs) are defined as single tumour cells or small clusters not larger than 0.2mm. These are normally detected or confirmed using immunohistochemistry. They are not considered to have any clinical significance and are given a pN0 in the TNM staging guidelines (Ellis et al, 2005).

There is still some controversy about the clinical significance of ITCs. The current consensus for the majority is for axillary dissection to be performed after the presence of micro- or macrometastases is identified (Bishop et al, 2009). However, several studies have been carried out investigating the likelihood of positive non-sentinel lymph nodes in cases where only ITCs were found in sentinel lymph nodes. The sentinel lymph node is the first node to receive lymphatic drainage from the primary tumour site (Mansel, 2006). A systematic review by Carolien H. M. Van Deurzen et al (2008) was carried out looking at 29 studies with a total number of 836 patients, of whom 108 had non sentinel lymph node involvement. The inclusion criteria included patients who had a diagnosis of primary invasive breast cancer and a positive sentinel lymph node with ITCs measuring less than 0.2mm. Patients who had had neoadjuvant chemotherapy were excluded from the study. It found that the pooled overall risk of non-sentinel lymph node involvement in patients with ITCs in the sentinel node was 12.3%, and of those 63.5% were macrometastases. It listed several characteristics for strong predictors of non-sentinel lymph node involvement including the size of the metastatic tumour in the sentinel node, a large primary tumour size, lymphovascular invasion and the presence of more than one positive sentinel lymph node (Degnim et al, 2003). In other studies it was found that in patients with sentinel lymph nodes containing micrometastases, approximately 20% will have metastases in non-sentinel lymph nodes (Cserni G et al, 2004 and Viale G et al, 2005 and Wada N et al, 2008). It has also been reported that the risk of false-negative sentinel lymph node results is approximately 7-8% (Lyman GH et al, 2005 and Goyal A et al, 2006). Therefore, in patients with ITCs in their sentinel lymph nodes, it could be argued that a wait and see policy might be acceptable. However, the 12.3% of patients who had negative sentinel lymph nodes but positive non-sentinel lymph nodes (63.5% of which were

macrometastases) in the study by Carolien H. M. Van Deurzen et al (2008) would have been undertreated if they had not had an axillary dissection. The management of ITCs still remains a contentious issue, however, for the time being while large-scale studies are still ongoing they are widely regarded as clinically insignificant.

1.1.8   Diagnosis and management of a breast cancer patient

A multidisciplinary approach is needed in order to diagnose and manage a patient with breast cancer. From the initial steps of diagnosis to surgical excision and follow-up care, there are several health-care professionals who are key to delivering a service tailored to the individual patient. The management of a breast cancer patient is summarised and the main discussion is centred on the concept of the sentinel node and the diagnosis of metastatic breast cancer and staging.

<u>Triple assessment</u>
Patients may present to the surgeon either having been referred from breast screening or from the GP for investigation of breast symptoms. Triple assessment is composed of three diagnostic modalities.
1. Imaging – mammography and/or ultrasound
2. Clinical examination
3. Histopathological with or without cytological examination

The main indications for patients to be recalled for assessment are those with a significant mammographic abnormality, significant breast symptoms or signs, review of short term recall or significant Magnetic Resonance Imaging (MRI) abnormality in women at high risk (Liston et al, 2010).

The following flow-charts summarise the general management of a patient with a breast lesion at each stage.

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```
                Screening                              Symptomatic
               (age 47 - 73)

              Mammography


    Normal (>93%)              Abnormal (<7% prevalent, <5% incident)


  Routine screening                Recall for assessment


                        Assessment (further imaging)
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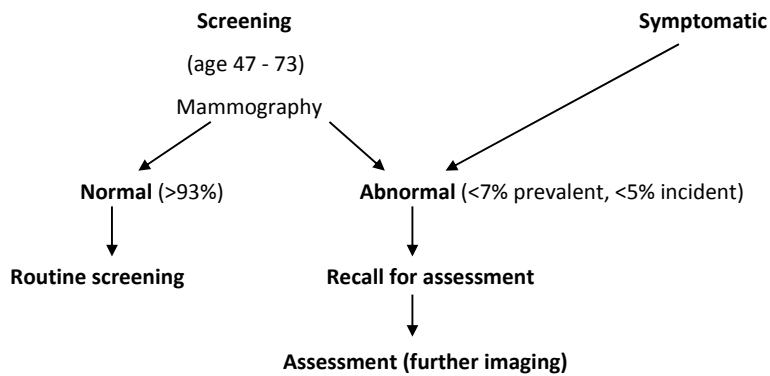
The flow chart illustrates that of the women who undergo mammographic examination at breast screening, more than 93% will have normal findings. However, a percentage will be found to have an abnormality detected on mammography and will therefore require further assessment in the form of further imaging (Liston et al, 2010).

```
                   Assessment (further imaging)


        Further Mammography              Ultrasound (US)
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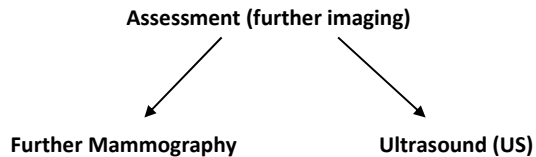
Further mammography
- To confirm the presence, morphology and site of the mass

Ultrasound (US)
- To establish the nature of the mass

There are four types of mammographic abnormalities described, namely masses, architectural distortion, asymmetric density and microcalcifications. Each of these abnormalities follow different management protocols but usually include needle sampling. Once further imaging has been carried out, the next step would be either to send the patient back to routine screening or to refer for a clinical examination.

Patients who are symptomatic automatically require clinical examination (Liston et al, 2010).

Assessment (further imaging)

Normal → **Routine screening**

Abnormal → **Clinical examination** ← **Symptomatic**
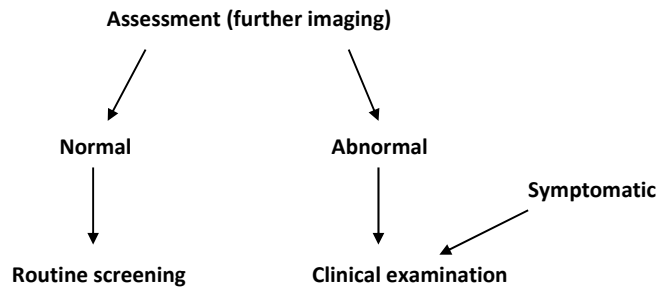
Clinical examination is mandatory for patients with a confirmed mammographic or US lesion that requires needle sampling. It is not necessary for those whose further imaging is normal (Liston et al, 2010).

Clinical examination

Normal → **Routine screening**

Abnormal → **Needle sampling** → **Multidisciplinary team meeting (MDT)**

Normal → **Routine screening**

Abnormal → **Treatment**
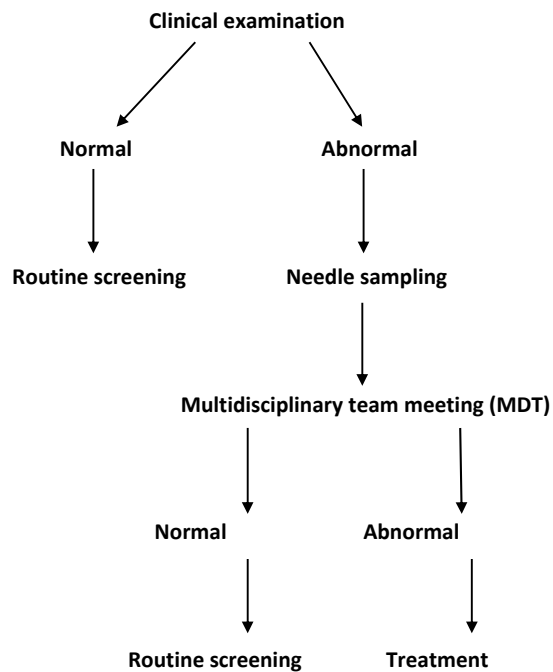
Needle sampling of the lesion can be done either as a core biopsy or as a vacuum assisted biopsy (VACB) depending on the type of lesion identified. Core biopsies provide information on tumour type, invasive status, grade and receptor status and can diagnose benign conditions. VACBs are recommended for lesions with microcalcification, after a B3 or B4 result at core biopsy and for diagnostic excision of some papillary lesions and radial scar/complex sclerosing lesion diagnosed on core biopsy (Liston et al, 2010).

In patients with invasive breast cancer, ultrasound of the axilla should be carried out to assess the possibility of metastatic disease. An axillary lymph node that is entirely hypoechoeic, has a cortex greater than 2mm, has a focal cortical bulge or a short-long axis ratio greater than 0.5 should be considered as potentially abnormal. Core biopsy and/or fine needle aspirate cytology (FNAC) should be carried out on the abnormal lymph node. A pre-operative diagnosis of metastatic disease in the lymph node will render sentinel lymph node biopsy as inappropriate (Liston et al, 2010).

All needle sampling results should be reviewed at the MDT meeting where the management of the patient is discussed with all the team members.

It is widely regarded that holding regular multidisciplinary team meetings is the best way to decide and co-ordinate patient management (Keeson, 2012). Each discipline involved in patient care should be represented. These can be divided into the diagnostic team and the cancer treatment team (Bishop, 2009).

Diagnostic team
- The breast surgical team including consultant, registrars or trainees
- Consultant Radiologist
- Consultant Histopathologist

Cancer treatment team
- Clinical Oncologist
- Medical Oncologist
- Breast cancer care nurse

- Research nurse
- Lymphoedema specialist
- Palliative care team
- Members of the diagnostic team

There are three main outcomes of the MDT. The patient could be reassured and discharged back to routine screening, referred for open surgical biopsy or referred for treatment. Decisions on further management and treatment options are discussed and a consensus reached. The patient is then informed of the decision, ideally by a clinician and a clinical nurse specialist, and their views discussed (Liston et al, 2010).

1.1.9 Surgical management

Once a diagnosis of breast cancer has been made, a care plan is drawn up and there is close communication between surgeon and oncologist. There are three broad categories the breast cancer diagnosis can be placed in.

1. Operable breast cancer

   Surgery is usually the first treatment. Neoadjuvant chemotherapy can be given to down-stage a tumour. Neoadjuvant endocrine therapy for Oestrogen receptor (ER) positive tumours may also be used to de-bulk the tumour and aid in breast-conserving surgery.

2. Locally advanced primary breast cancer

   In these circumstances, initial treatment with chemotherapy or radiotherapy may be appropriate before surgery.

3. Metastatic breast cancer

   At this stage, the main aim is palliation and to improve quality of life. Life expectancy is approximately two years. Oncologists and palliative team members are more involved in the management of these patients. (Bishop et al, 2009).

The type of surgical procedure depends largely on the size and extent of the tumour. Whether breast conservation surgery or mastectomy is chosen depends on the results

of clinical examination, US and mammography findings and on patient choice. MRI is also used in certain situations where invasive lobular carcinoma is suspected, the breast tissue is dense or the clinical and radiological assessment of extent disagree. For patients undergoing breast conservation surgery, the margins of excision are very important. The surgeon needs reassurance that the tumour has been completely removed from the patient and relies on the histopathological examination of the margins to confirm this. The breast specimen is orientated to allow the pathologist at cut-up to block the correct tissue to assess the margins. There are no nationally accepted guidelines on the minimum distance of tumour to the excision margin. There should be locally determined acceptable margin widths and each case should be discussed in multi-disciplinary meetings to assess the need for further excision to obtain clear margins if deemed appropriate (Bishop et al, 2009).

The most powerful prognostic determinant in breast cancer is the presence of axillary node metastases (Veronesi, 1997). A pre-operative assessment of the axilla involves ultrasound assessment with or without FNAC and a core biopsy to diagnose the presence of metastases. A positive result would lead to an axillary clearance. This would involve all the lymph nodes taken from the axilla. In over 90% of patients this would mean at least 10 nodes (Veronesi, 1997). The majority of axillary staging should be done by sentinel node biopsy, however, there may be some situations where axillary sampling (at least four nodes) or axillary clearance may be used. The policy in place at Princess Alexandra Hospital is for patients to undergo sentinel node biopsy for axillary staging.

1.2   Sentinel lymph node

In metastatic disease, the first step in the vast majority of breast cancers is spread to the ipsilateral axillary nodes via the lymphatics. In 98% of metastatic cases, the cancer is known to spread to the first, then second and then third level (Veronesi, 1987). Historically, the surgical management of breast cancer would have involved removal of the tumour either by breast-conserving surgery or mastectomy and staging of the cancer by removal of the lymph nodes in an axillary clearance. Clearing all or most

axillary lymph nodes carries with it a high risk of morbidity and poor quality of life in 8-15% of patients (Cserni, 2000). There are no therapeutic benefits of axillary lymph node dissection in node negative patients. Patients who were found to have screen detected breast cancer were generally found to have smaller, less advanced tumours and therefore a higher rate of node negative cancers (pN0) (Cserni, 2000). A solution was needed to avoid overtreatment of node negative patients.

The concept of the sentinel lymph node biopsy (SLNB) was born out of this need to avoid unnecessary overtreatment in node-negative patients who had undergone axillary dissection. A sentinel lymph node (SLN) is the first node to receive lymphatic drainage from the primary tumour site (Mansel, 2006). It relies on the theory that the spread of mammary carcinoma through the lymphatic vessels follows an orderly pathway (Cserni, 2002). The sentinel lymph node concept was first investigated in penile carcinoma. The investigators used lymphangiograms via the dorsal lymphatics of the penis to locate the presence of the sentinel lymph node. In 15 patients found to have a positive sentinel lymph node, 12 patients had negative clearances, demonstrating that the sentinel node is the first node to receive tumour cells, showing that all lymphatic channels draining into the iliac lymph nodes would always first drain into a sentinel lymph node. The study concluded that if the sentinel lymph node was found to be negative, then a clearance of all the nodes was unnecessary (Cabanas, 1977). They showed that there is a hierarchical organisation through which lymph flows in a systematic order. Therefore, the identification of the first lymph node the lymph drains into is the first step. The further management of the patient would depend on whether the sentinel lymph node was positive or not. Cutaneous malignant melanoma was the first cancer in which SLNB first altered staging and management. Breast cancer was the second type.

Over 40,000 new breast cancer cases per annum are diagnosed in the UK of which 25-30,000 will be suitable for SLNB (CRUK, 2012 and Kumar et al, 2010). The SLN is successfully localized in 99% of patients using a combined technique of radioactive (TcM99m) labelled nanocolloid and 2mls of diluted Patent V blue dye. (Garcez and Harper, 2009). The combined use was shown to be the most accurate in identifying the

sentinel node. Thirty-nine peer-reviewed pilot studies were carried out to report the validity of the sentinel lymph node with following axillary dissection as confirmation. Fifteen studies used radioisotope alone, twenty studies used blue dye alone and eleven studies used both. The identification rates were 92%, 81% and 93% respectively with false negative rates of 7%, 9% and 5% respectively. The lowest false-negative rate of 5% was attributed to the increased likelihood of more than one sentinel node being picked up by the combination method (Bonnema, 2002). The NEW START programme is a national SLNB training programme for surgeons who will be carrying out the procedure (Garcez and Harper, 2009).

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1.2.1   Sentinel Lymph Node Biopsy vs Axillary Clearance

There are two important criteria for SLNB to be preferable to axillary clearance (AC). The first is the reduced impact SLNB has on patient quality of life. The second is the concordance between the two procedures. The SLNB approach would not be adopted if the local recurrence of axillary metastases is unacceptably high. There have been numerous studies showing the level of concordance is high. Veronesi et al (1997)

identified the SLN in 160 out of 163 patients. Out of those 160 patients, 81 had positive SLNs and 79 had negative SLNs. Of the SLN positive patients, 32 were found to have only the sentinel node positive and 49 had other positive axillary nodes as well as the sentinel node. Of the SLN negative patients, 75 had negative axillary lymph nodes and 4 had positive axillary lymph nodes. Therefore, 5% of patients with negative SLNs were found to have positive axillary lymph nodes, a small proportion of the 160 patient population.

Naik et al (2004) found an extremely low rate of axillary recurrence in SLN-negative patients of 0.12% (3/2340). The conclusion reached in both studies was that a SLN-negative result would justify not performing an axillary clearance. A meta-analysis of 19 studies comparing SLNB with AC in early breast cancer patients from 1996-1999 was performed by Fraile et al (2000) in which the sensitivity ranged from 83% – 100% and the pooled data from the meta-analysis gave a global sensitivity of 91%. They concluded that SLNB was a practical alternative to AC.

The ALMANAC trial is a multi-centre randomized trial that compares the quality-of-life (QOL) outcomes between patients who underwent SLNB and AC. The trial assessed the known side effects of axillary dissection including:

- Lymphoedema: a well known complication seen in 10-20% of patients
- Arm numbness/sensory deficit
- Impairment of shoulder movement: most affected is flexion and abduction

It was found that in all three complications, SLNB was associated with less arm and shoulder morbidity than AC. Patients who had had SLNB returned to normal domestic activity sooner than AC patients. Other benefits of SLNB were the reduction in healthcare costs associated with lymphoedema. AC patients undergo wound drainage. By having a SLNB the need for wound drainage is eliminated which reduces the number of clinic visits and reduces costs. Also, the length of inpatient stay for patients who had undergone AC was longer than those with SLNB. The study concluded that SLNB is a safe and effective alternative to routine AC for staging breast cancer. SLNB

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was associated with less arm morbidity and better QOL and also reduced costs to the healthcare service (Mansel, 2006).

Patients found to have positive sentinel lymph nodes undergo AC. However, there have been few studies assessing the outcome of patients with positive sentinel lymph nodes who did not go on to have ACs. The Z0011 trial (Giuliano et al, 2011) is a prospective trial that examined the survival of patients with positive SLNs who did not have axillary clearances compared to those that did. The primary end point was overall survival and the secondary end point disease-free survival. The inclusion criteria included women over 18 years of age with clinical T1 or T2, N0, M0 breast cancer. All patients had lumpectomies and had their positive SLNs confirmed by either frozen section, touch imprint cytology or permanent section. AC was defined as level I and II dissection with a minimum 10 lymph nodes. Exclusion criteria included patients with three or more positive SLNs, as well as those with gross extranodal disease, positive lumpectomy margins and metastases detected by Immunohistochemistry only. Over 95% of patients received adjuvant systemic therapy (both chemotherapy and hormonal therapy) and all had whole breast irradiation. Of the planned 1900 patients with final analysis after 500 deaths, only 891 patients were enrolled as the study closed early due to a lower than expected mortality rate. 445 patients were randomized to have AC and 446 to SLN dissection only. At a median follow up of 6.3 years, it was found that those in the SLN-only arm did not have a statistically inferior overall survival than those in the AC arm. The five-year overall survival rates were 92.5% (95% CI, 90.0% – 95.1%) and 91.8% (95% CI, 89.1% - 94.5%) in the SLN-only arm and AC arm respectively. Disease-free survival also did not differ significantly with a five-year disease-free survival rate of 83.9% (95% CI, 80.2% - 87.9%) and 82.2% (95% CI, 78.3% - 86.3%) in the SLN-only arm and AC arm respectively. In summary, the trial found that women with a positive SLN and clinical T1-T2 tumours that have undergone breast conserving surgery with radiation therapy and systemic therapy do not benefit from AC in terms of local control, overall survival and disease-free survival. The discovery of further positive lymph nodes in an AC does not in the majority of cases affect further treatment and as AC is associated with a significant increase in

morbidity, the results raise the possibility of eliminating the need for AC in patients who match their inclusion criteria.

1.2.2 Histopathological assessment of the sentinel lymph node

Locating the SLN is the crucial first step carried out by surgeons using the combined technique described previously (section 1.2). The next crucial step in the evaluation of the SLN is the presence or absence of metastasis determined by histopathological examination. Since there is usually one or a few nodes submitted for pathological examination, it has allowed a more vigorous and concentrated pathological effort on staging.

There is no consensus on the histopathological assessment of SLNs nationally and in Europe. A European questionnaire-based survey carried out by the European Working Group for Breast Screening Pathology (Cserni et al, 2004) found that out of 240 institutions surveyed, there were 123 different protocols used in the histological assessment of the SLN. The methods ranged from using one H&E stained level for each block to performing multilevel sectioning for each block to using both multilevel sectioning and immunohistochemistry (IHC). The distance between each step also varied from 2μm to 250μm. The IHC marker employed was mainly a pan-cytokeratin marker, usually AE1/3 or MNF116. However, other markers such as CK7, CK8, ER, PR and EMA were also used (Cserni, 2004). Several studies have shown that using a combination of multiple level sectioning and IHC would provide the lowest false-negative rate (Torrenga et al, 2001). Some investigators have shown that by re-examining axillary lymph node dissection (ALND) specimens by step sectioning, 8-13% of previously node-negative patients were found to have positive nodes. Turner et al (2001) found that by using a combination of Haematoxylin and Eosin staining (H&E) and IHC, 20% of patients previously thought to be node negative were found to be node-positive. However, the workload associated with complete serial sectioning would be unreasonable, especially in a busy department with limited resources and staff. An acceptable and realistic workload which did not jeopardise sensitivity is the optimum goal. There have been several studies trying to establish the optimum

number of levels and the interval between them (Cserni, 2004, 2002, 1999 and Torrenga et al, 2001). All agree that the number of SLNs found positive increased with each new level and that the percentage of micrometastases and ITCs increased when using IHC. The NHS breast screening recommendations as stated in the 'Pathology Reporting in Breast Cancer Screening' report (2005) are that block dissection techniques can be an alternative to high numbers of serial sectioning. It recommends the following:

- For lymph nodes greater than 5mm: not more than 3mm slices taken perpendicular to the long axis
- For lymph nodes less than 5mm: bisected or alternatively embedded whole

Further assessment techniques carried out include multiple levels and immunohistochemistry, however, these are not recommended in routine practice due to the significant resources required. The report acknowledges that the universally accepted optimum way of examining SLNs is still under discussion and that research is ongoing.

An important point to note is that while IHC staining using a pancytokeratin marker has been shown to increase sensitivity, the specificity can be variable. Pitfalls associated with IHC and in some cases morphology as well include:

- Mistaking rare benign epithelial inclusions for tumour such as benign glandular, apocrine or squamous epithelium as illustrated in figure 4. However, these are found in less than 1% of SLNs and can usually be identified by morphology

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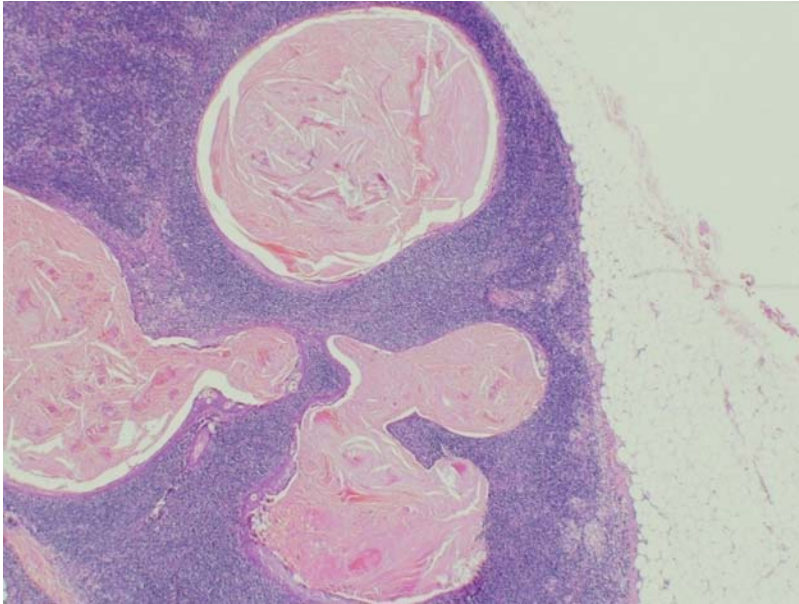
Figure 6. Epithelial inclusion cysts within a lymph node (x10). The epithelial component is positive for pancytokeratin markers.

- Mistaking positive dendritic reticulum cells or macrophages for tumour cells. In most cases, cellular morphology can help distinguish these from tumour cells.
- Staining artefacts which can appear dot-like but are not associated with morphology
- Carryover from cutting blades, prior needle biopsy or other surgical interventions

It is recommended that IHC positivity should be correlated with morphology (Cserni, 2006 and Turner et al, 2001).

SLNB has been widely accepted as the preferred choice of axillary staging in patients with clinically negative lymph nodes. Those patients who had a negative SLNB were spared the complications of an AC and those patients who had a positive SLNB would

have a second operation to remove the axillary lymph nodes. This was accepted as best practice, however, it soon became clear that an intra-operative diagnosis would be more beneficial. It would allow patients with a positive SLN to undergo an axillary clearance during the same operation, thus avoiding the prospect of a second operation.

1.2.3   Current intra-operative techniques

Two techniques for the intra-operative diagnosis of sentinel lymph nodes are frozen section (FS) and touch imprint cytology (TIC). There have been many studies comparing the sensitivity and specificity of each technique in assessing sentinel lymph nodes (Salem et al, 2002, Chilosi et al, 1994, Liu et al, 2011, Lumachi et al, 2012, Menes et al, 2003, Mori et al, 2006). Each test has its own advantages and disadvantages. Attempts to increase the accuracy of diagnosis have been tried including combining the two methods and using rapid staining for cytokeratins. However, there still remains no internationally accepted standard for the intra-operative assessment of sentinel lymph nodes.

1.2.3.1   Frozen section (FS)

This method involves freezing the tissue in order to take thin slices to mount on a slide. A cryostat is a microtome in a freezer which is capable of slicing tissue 1μm thick. The specimen is placed on a metal disc and the tissue frozen to between -20°C to -30°C. A gel consisting of polyethylene glycol and polyvinyl alcohol is used to embed the frozen tissue. The frozen tissue is then cut with the microtome, mounted on a slide and stained with haematoxylin and eosin (H&E) and examined. The whole process takes approximately ten minutes.

The advantages of this method includes allowing:
- A tissue diagnosis and architectural information
- A distinction between micro- and macrometastases

There are common disadvantages to using this method of intra-operative diagnosis including:

- Freezing artefacts hindering diagnosis
- Loss of tissue
- Being expensive to run
- Being time-consuming in inexperienced hands
- Relying on a trained biomedical scientist (BMS) and experienced Consultant Histopathologist to be on call
  (Cserni, 2006)

1.2.3.2 Touch Imprint Cytology (TIC)

TIC involves dabbing the slice of tissue onto a glass slide, which is then immersed into a 90% alcohol solution and stained using the Papanicolaou method.

Advantages of TIC:
- It is cheap
- The method is fast and easy to carry out

The disadvantages to this method include:
- No architectural morphology can be assessed
- Relying on an experienced consultant histopathologist to be on call
- Assessing fewer cells than other methods
- Rarely differentiating between macro- and micrometastases
- Cells such as histiocytes and follicle centre cells being misinterpreted or suspected of being atypical and therefore having a C3/C4 diagnosis, a deferred diagnosis (Cserni, 2006).

1.2.3.3 Frozen Section vs Touch Imprint Cytology

Comparisons have been made between FS and TIC with different results reported. However, the majority of findings suggest that FS has a better sensitivity and a marginally better or comparable specificity. A meta-analysis of nine studies comparing TIC and FS included a range of cases examined from 20 to 429. The analysis showed that TIC sensitivity ranged from 47.1% to 98%, compared with FS which ranged from 52% to 90.2%. The overall sensitivity was found to be higher with FS. The specificity of both FS and TIC were high, averaging 99% to 100% with only one study reporting a 90.8% specificity with TIC (Mori et al, 2006).

A study comparing FS, TIC and a combination of both showed that TIC used on its own was the least sensitive and that FS was better. However, combining the two techniques showed had an even better sensitivity (Lumachi et al, 2012).

A large meta-analysis was carried out to determine the accuracy of FS results. 47 studies were included with a total of 13,062 patients with 32% having positive sentinel lymph nodes. Summary Receiver Operating Characteristic (SROC) curve analysis was applied to account for the differences between each study where the threshold for defining a positive result may be different. It found that the mean sensitivity for detecting metastases using the SROC model was 73% and the mean specificity 100%. 18 studies compared FS in macro- and micrometastases. It found that the mean sensitivity for detecting macrometastases was 94% compared to the sensitivity of micrometastases of 40%. It concluded that for macrometastases, it was a valid test with a high sensitivity. However, it could not be relied upon to diagnose the majority of micrometastases (Liu et al, 2010).

The accuracy of TIC is even more variable than FS. A meta-analysis of 31 studies evaluating TIC showed the pooled sensitivity to be 63% and specificity 99%. For macrometastases, the pooled sensitivity was 81% but the pooled sensitivity of micrometastases was only 22%. It found that although TIC is fast and cost effective with a high sensitivity for macrometastasis, it does not have a good sensitivity for micrometastases (Tew et al, 2005).

IHC staining in an intra-operative setting was dependant on the length of time taken to stain the slide. A rapid immunostaining method was developed using the enhanced polymer one-step staining system. Primary antibodies and horseradish peroxidise is linked to dextran, a chemically inert polymer complex. A few minutes of incubation at room temperature was found to be enough to provide sufficient immunostaining (Tew et al, 2005 and Bisgaad et al, 1993).

A prospective study comparing TIC, FS and rapid cytokeratin immunostaining (RCI) was carried out on 100 patients with 297 sentinel lymph nodes. 20 patients were found to have positive sentinel lymph nodes, 12 with macrometastasis and 8 with micrometastasis. TIC, FS and RCI were compared singly as well as a combination of TIC and FS and a combination of FS and RCI. The H&E section was taken to be the gold standard. The results showed that TIC used on its own was the least sensitive with a value of 50%. It was found to be particularly bad at detecting micrometastases, identifying only 1 case out of 8. However, the specificity was 100%. FS on its own was the next least sensitive with 72.2%, detecting all 12 macrometastases but failing to find 5 micrometastases. RCI had a sensitivity of 77.8% missing 4 micrometastases. This sensitivity was the same as combining TIC with FS. The most sensitive combination was that of FS and RCI achieving 83.3%. The specificities of the last four tests were found to be the same (97.5%). The study concluded that FS and RCI were the most sensitive diagnostic tool (Krishnamurthy et al, 2008).

By comparing the different intraoperative tests, it is clear that there is no consensus on the best method, with some authors reporting wildly different sensitivities and specificities for each test singly and in combination. This discrepancy is related to the variations of the studies. These variations include the study design, the number of patients included, the type of breast cancer they had and how many of them had positive nodes. Other aspects that would have led to variation include the experience of the pathologist, the skill of the technicians or BMS staff in preparing the FS and TIC slides and the ability of the surgeons in recognising and excising the sentinel lymph node (Safai and Razeghi, 2012).

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An alternative to the current methods of FS and TIC is the use of molecular diagnostic technology. There have been rapid advances in molecular pathology related to breast cancer providing an alternative to conventional histological and cytological assessment.

1.3 Molecular pathology and its application in breast cancer

Molecular pathology uses the techniques of molecular biology to enhance the understanding, diagnosis and therapy of disease. It is the testing of nucleic acids within a clinical context (Tubbs and Stoler, 2009). Molecular pathology is a rapidly expanding field and is fast becoming an everyday tool for diagnostic and prognostic purposes. The impact and potential advantages of molecular pathology in breast cancer has been growing at an exponential rate (Weigelt et al, 2010).

The growing number of treatment options including hormonal therapies, combination chemotherapy regimens and targeted treatment options such as Herceptin for Her2-positive patients have highlighted the need for a way to divide patients according to the likelihood of disease recurrence after completion of local therapy. Identifying patients more likely to develop disease recurrence so that the best treatment can be given is an aim that needs to be met. There are morphological indicators of prognostic value that the histopathologist can record, as well as the subtype of cancer. However, it is known that there is considerable molecular heterogeneity between each type of cancer and between each patient. These variations will make an important impact on the response to therapy the patient receives. They can be identified by the gene expression profile of the tumours, ie which mRNAs are being made and in what quantities (Weigelt et al, 2010).

1.4 Gene expression

The control of gene expression is complex, involving diverse and specific processes that can either activate or repress expression. The control of expression occurs in the transcriptional and post-transcriptional levels, allowing a gene to be either 'turned on' or 'turned off'. Transcription factors are proteins that mediate transcription control.

Some factors can increase the rate of transcription by binding to enhancer sequences while others can repress transcription by binding to 'silencer' sequences in the DNA. Post transcriptional control of gene expression affects various processes including export of the mRNA from the nucleus to the cytoplasm, mRNA stabilization and mRNA degradation. Other types of RNA have emerged as important regulators of reducing gene expression. These include micro RNA (miRNA) and short interfering RNA (siRNA). They are both between 21 and 26 nucleotides long and function to inactivate specific mRNAs in sequence specific manners. They do this by binding to target mRNA sequences using a protein complex called the RNA induced silencing complex (RISC). When siRNA binds to RISC, the target mRNA is cleaved and degraded. When miRNA binds to RISC, it base pairs with the 3' untranslated region of the mRNA, preventing translation. The regulatory pathways that these short RNAs mediate are called RNA interference or RNA silencing (Watson et al, 2007).

1.4.1 Gene expression profiling

Molecular gene expression profiling using array technology has expanded knowledge of the biological and clinical diversity of breast cancer. Gene expression profiling allows for the analysis of the expression of thousands of genes at once, giving a global picture of cellular function. All cells contain the same genome, however, not all genes are expressed. It is this selectivity in turning genes 'on' or 'off' that differentiates one cell type from another. Gene expression control occurs at the transcriptional and post-transcriptional level, that is whether to make the mRNA of a certain sequence coding a specific protein and how much of it (Watson et al, 2007).

There are several methods of carrying out gene expression profiling, including serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS), oligonucleotide arrays and complementary DNA (cDNA) arrays, the last two being the most commonly used (Pollock, 2002). A microarray is a solid surface, either made of plastic, glass or silicon, on which is a 2-dimensional array of compartments that are accessed by their position in the array. An array is defined as a set of items that are randomly accessible by numeric index (Bruns et al, 2007). DNA microarrays rely on the

property of mRNA to hybridise with its cDNA. On the solid surface, known DNA fragments are denatured and as many as several million are deposited in an orderly array on the solid surface by robotic machines. mRNA is extracted from normal tissue and tissue containing tumour and reverse transcribed to cDNA. During the process, fluorescent nucleotides are added and different ones are used in both tissues, for example red dye for the tumour sample and green dye for the normal tissue. The labelled cDNAs are then bathed onto the surface and each one hybridises to its known complementary DNA fragment. A special scanner is then used to measure the intensity of the fluorescence for each labelled cDNA. The amount of fluorescence of each particular gene corresponds to the amount of mRNA being produced by the sample, ie whether that gene is being expressed and how much. The more active a gene, the more intense the fluorescent signal. The masses of data generated is analysed using sophisticated biostatistical and bioinformatics methods such as hierarchical cluster analysis. This involves ranking the genes according to how similar their gene expression levels are. To visualise this information easily, a heat map is generated whereby each data point is shown as green or red. If a spot is red, it means the gene is overexpressed in the tumour. If it is green, it is underexpressed and if it is yellow is equally expressed in both tissues. In this way, the gene expression profile of the tumour is known, highlighting genes which are over or under expressed (National Human Genome Research Institute, 2011). Three fields of discovery are introduced when analysing the information from these gene expression profiles (Weigelt et al, 2010).

Class discovery studies and Class comparison studies

Discovery studies are where microarrays are used to identify distinct subgroups in an apparent homogenous series of samples. These subgroups are then analysed with specific correlations in mind, for example, with disease progression. Class comparison studies compare two or more known gene expression profiles to identify the molecular differences between the groups.

Four molecular subtypes have been identified in breast cancer (Perou et al, 2000). These are the positive oestrogen receptor (ER) cancers luminal A and luminal B and the ER-negative cancers Her-2, basal-like and normal breast-like cancers:

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- Luminal: This group can be further subdivided into luminal A and luminal B. These are ER positive cancers that show similar expression patterns to those of normal luminal epithelium cells. Luminal A cancers usually have low histological grades, lower proliferation rates and a good prognosis compared with luminal B cancers which usually have a higher histological grade, higher proliferation rate and a worse prognosis.

- Normal breast-like cancers: these are still poorly characterised and are shown to have gene expressions similar to those expressed by adipose tissue. Fibroadenomas and normal breast samples are clustered together in this group.

- Her-2: These tumours overexpress Her-2 and are associated with high histological grade and a poor prognosis.

- Basal-like: these cancers are shown to express genes normally expressed by basal or myoepithelial cells. These tumours are also typically triple-negative i.e. negative for ER, progesterone receptor (PR) and Her2, and are characterised by high histological grade, have high mitotic indices, central necrosis, pushing tumour borders, conspicuous lymphocytic infiltration and have atypical/typical medullary and metaplastic-like features. These tumours show a high response rate to chemotherapy treatment (Perou et al, 2000).

It is now thought that breast cancer is a heterogenous disease constituting multiple diseases affecting the same site. Molecular classification of breast cancer is still in the early stages and the subjective nature of how the subtypes were discovered, the stability of each subtype and the lack of reproducibility of the subgroups limits molecular classification for diagnostic purposes. Until an internationally accepted molecular subtype classification is agreed on, the applicability of this methodology to patient management remains limited.

Class prediction studies

Once the transcriptional or molecular differences between two or more groups are identified, a 'gene signature' can be defined for each group in order to predict the class of a new sample. Examples of gene signatures for breast cancer include the MammaPrint® 70 gene signature and the Oncotype DX®.

MammaPrint® is a 70-gene assay used as a breast cancer multigene classifier (Gokmen-Polar and Badve, 2012). It has been found to successfully predict disease outcome (Dobbe et al, 2008). The patients in the initial analysis comprised 78 patients younger than 55 years with breast cancers measuring less than 5cm and with negative lymph nodes. By comparing the gene expression profiles of those patients who developed metastasis within 5 years (poor prognosis) with those who did not (good prognosis), a prognostic signature of 70 genes was identified. Patients who were grouped into the good prognosis category could be spared the toxic effects of chemotherapy. The signature has since been validated in various retrospective studies and was shown to outperform other established systems based on clinical and histological parameters in predicting disease outcome (Buyse et al, 2006, Mook et al, 2009, Bueno-de-Mesquita et al, 2009). MammaPrint® has received Food and Drug Administration (FDA) approval to be offered as a prognostic test for breast cancer patients. The inclusion criteria for these patients are those aged less than 61 years, have tumours measuring less than 5cm, to be node negative and to be stage I/II. The drawbacks with this methodology is its limited clinical use in ER-negative breast cancers as only 0-4% of patients with ER-negative breast cancers are considered to have a good prognosis using MammaPrint®. Patients with Her-2 positive breast cancer (5-22%) were shown to have a good prognosis 70 gene signature, however, withholding appropriate anti-Her-2 therapy would be considered controversial. Finally, none of the validation studies were performed using randomized clinical trial populations. Despite these drawbacks, proponents of MammaPrint® state that the results could be used as a replacement for clinicopathological parameters (Weigelt et al, 2010).

Microarray experiments provide crucial information about tumour biology; however, they are too cumbersome to be used in day to day work in the laboratory or clinic and unlikely to be used for clinical work. Therefore, identifying a smaller subset of the most important, clinically relevant genes and testing for them in a quick and easy assay is the most desirable outcome. Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) is a molecular tool that allows clinicians to do just that. By using formalin-fixed paraffin-embedded breast cancer tissue, RT-qPCR can be used to quantify the

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expression of clinically relevant tumour-related gene transcripts. A more detailed description of the PCR methodology is given in section 1.6. An important example of this is the Oncotype DX®, a validated assay that detects 5 reference genes and 16 tumour-related genes. These were chosen from 250 candidate genes that were selected by various methods including literature searches, and cDNA microarray analysis. These 16 genes were found to have a consistently strong correlation with the likelihood of disease recurrence when tested on 447 breast cancer patients with ER positive tumours and negative lymph nodes. By using an algorithm based on the genes' level of expression, a recurrence score (RS) was calculated for each breast sample. Three risk groups were identified: low risk (<18), intermediate risk (18-31) and high risk (>31). The RS has been shown to be consistently accurate in several studies.

Further studies have shown that the RS is predictive for tamoxifen efficacy and for response to chemotherapy. Those patients with a low RS receive the greatest benefit from tamoxifen therapy and are unlikely to gain any further benefit from chemotherapy. Those patients with a high RS benefit most from chemotherapy. Therefore, patients with a low RS can be assigned hormonal therapy only and those with a high RS can receive both hormonal and chemotherapy. In this way, Oncotype DX® can triage patients based on their molecular expression of these 16 genes into treatment groups (Dobbe et al, 2008, Tubbs and Stoler, 2009). Oncotype DX® has also been approved for use by NICE as a cost-effective option to guide the treatment decision of patients with early breast cancer and with an intermediate risk of distant recurrence (NICE, 2013).

Another RT-qPCR based assay is Theros®. The gene expression profiles of 60 patients treated with adjuvant tamoxifen were analysed using microarrays. Three strongly predictive genes were identified: homeobox gene HOXB13, interleukin 17B receptor (IL17BR) and EST AI240933. The expression ratios between HOXB13 and IL17BR strongly correlated with recurrence, outperforming other clinical and pathological predictors. After further retrospective validation studies, the HOXB13:IL17BR ratios were shown to be a predictor of outcome for ER-positive patients treated with surgery alone and with negative lymph nodes. The test defines the risk of recurrence and benefit from endocrine therapy (Ma et al, 2004, Weigelt et al, 2010).

Lastly, MapQuant Dx® is an 8-gene RT-qPCR assay used to determine the grade of breast cancers. Histological grading is subject to varying concordance between different pathologists, in the range 50% - 86% (Robbins et al, 1995). It was shown that histological grade I tumours had a distinctive gene expression pattern to histological grade III breast cancers (Ma et al, 2003). Sotiriou et al, 2006, tried to define the molecular basis of histological grading by performing microarray tests on 33 and 31 histological grade I and grade III ER-positive breast cancers respectively. A 97-gene expression grade index (GGI) composed of genes related to cell cycle and proliferation was identified. GGI was shown to have a strong association with histological grade when applied to a dataset. GGI was also shown to have a stronger association with relapse-free survival than histological grade. The assay could also stratify histological grade II tumours into GGI low grade and GGI high grade which correspond to grade I and grade III cancers respectively. GGI is shown to be an independent prognostic factor and a predictor of outcome in tamoxifen-treated patients (Loi et al, 2007).

In summary, the level of understanding of the genetic mutations and pathway changes that lead to breast cancer is expanding. New insights into the clinical course of different subtypes of tumours and recognising new predictive and prognostic markers for response to targeted therapies is a major advantage in the molecular profiling of breast cancer. The prognostic and predictive signatures provided by MammaPrint®, Oncotype DX®, Theros® and MapQuant® still need to be validated using larger datasets in prospective studies before they are accepted into clinical decision-making. However, the information provided by microarrays and the prognostic and predictive gene signatures produced promise to become invaluable in the trend towards tailoring individual therapy by recognising patients with a good prognosis in whom chemotherapy and its toxic side effects could be omitted as well as recognising predictive therapeutic markers that could identify patients who would benefit from chemotherapy (Weigelt et al, 2010).

1.4.2   Nucleic acid extraction, purification, isolation and quantification

All molecular pathology tests rely on nucleic acid extraction, purification and isolation. Some also require quantification of the nucleic acid (Tubbs and Stoler, 2009).

Nucleic acid extraction

Cell lysis starts the process of nucleic acid extraction. The kind of extraction depends on several factors, including the type of tissue, desired purity of the nucleic acid and the downstream application. Detergents or enzymatic digestion can be used to cause chemical disruption of the cells. Mechanical disruption can involve sonication or homogenization. Fresh solid tissue should be homogenized in an appropriate buffer first before sonicating.

Purification and isolation of nucleic acids

There are several different ways of purifying and isolating nucleic acids. These include silica binding, anion-exchange chromatography and magnetic bead binding. Silica binding is based on the known affinity nucleic acids have to bind to silica in a high salt concentration. By applying binding and wash buffers, nucleic acids can be separated from their cellular components. This is the method of isolation used in many commercially available products, including the RNeasy silica membranes in the spin columns from Qiagens RNeasy kit. Anion-exchange chromatography uses columns that are made of an anion-exchange resin that binds the nucleic acids by attracting the negatively-charged phosphate backbone of the nucleic acid to the positively charged residues in the resin. The magnetic bead binding technique binds magnetic particles to nucleic acids which are then pulled to the side of a tube by magnetic currents while the cellular components and debris are washed away.

Nucleic acid quantification

Nucleic acid quantification can be measured using a spectrophotometer. The device measures the absorbance of the nucleic acid solution at several wavelengths of ultraviolet light. Nucleotides have a maximum absorbance of 260nm (A260) and proteins of 280nm (A280). The ratio of A260:A280 gives an estimate of the purity of the sample. Pure RNA has a ratio of 2.0. Protein contamination would lead to a lower ratio.

Electrophoresis is a method used to identify DNA based on their molecular weight. The DNA is loaded onto a liquid or gel with an electric field. Agarose gel is commonly used and the concentration depends on the number of base pairs the DNA targets have. 1% concentration agarose gels are used to separate DNA composed of 1-20kbp and the higher concentration gels are used to separate smaller fragments of DNA. As DNA is negatively charged, it migrates towards the positively charged end of the field, the anode. The mobility of the DNA molecule is inversely proportional to the log of its size. Therefore, smaller molecules travel through the gel quicker. Other factors influencing mobility include the net charge of the molecule, temperature and pore size of the matrix. By adding ethidium bromide, the DNA can be visualised when illuminated by ultraviolet light. Ethidium bromide molecules are planar and can intercalate between the bases of double stranded DNA (dsDNA). By exposing the gel to UV light, the ethidium bromide-stained DNA fragments fluoresce. By comparing the location of the DNA to DNA with known molecular weights in a nearby lane of the gel, the molecular weight can be determined. The quality of the DNA is confirmed by a sharp band that implies high-quality, intact DNA. Smeared DNA with a thicker band extending down the well implies DNA degradation has taken place (Tubbs and Stoler, 2009).
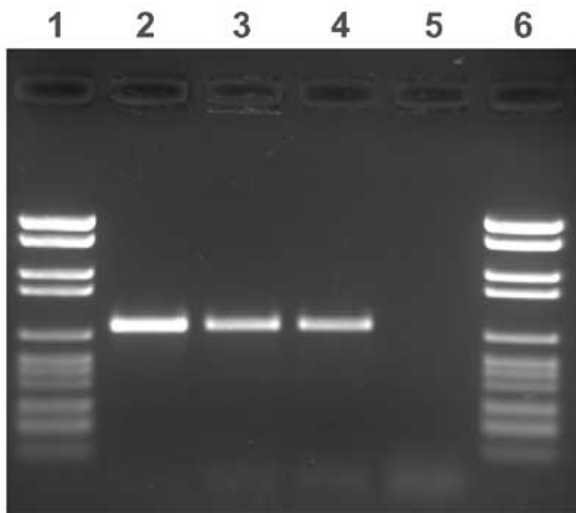
Figure 7. The figure shows a gel with marker weights on either side with bands corresponding to known masses (base pairs) and three end products of a PCR reaction. The three bands are compared to the marker weight 'ladder' and the mass confirms the presence of the desired PCR product (Alaska BioPREP, 2011).

It is important to note the difference in stability between RNA and DNA when extracting either one. DNA is highly robust due to the instability of DNase enzymes and is far less reactive than RNA due to the absence of the –OH group in deoxyribose. In contrast, RNA is easily degraded by RNase enzymes present in the cell. RNases are stable and can regain function after denaturation. Therefore, during the process of RNA extraction, cellular RNases should be inhibited and degraded as soon as possible with the addition of either guanidine isothyocyanate (GITC) or beta-mercaptoethanol. GITC is also used in spin columns that use silica based membranes to promote solubility of non-polar proteins in water, promoting adsorption of nucleic acids to the spin column (Tubbs and Stoler, 2009).

1.4.3   The use of PCR in clinical practice

The polymerase chain reaction (PCR) is a molecular tool used to amplify targets of DNA. This is done by designing primers – short DNA sequences – that bind at the beginning and end of the desired DNA target. The DNA is added to a mixture containing primers, free nucleotides and DNA polymerase, an enzyme involved in DNA synthesis. A thermocycler is used to heat and cool the mixture at temperatures that allow PCR to take place. Details of how PCR works is given in section 1.6.2.

There are many applications of PCR in a variety of areas including detecting known specific genetic variants in pathological conditions and detecting the presence of new targets for therapy such as chromosomal rearrangements or micro-organisms. A tumour's genetic mutation or chromosomal abnormality can be detected by PCR and used as a disease-specific marker. That information can be used to direct cancer treatment by appropriate selection of chemotherapy, aid in assessing disease remission, monitor the patient during therapy and provide prognostic information.

Several important examples of applications of PCR in the clinical setting are briefly described (Tubbs and Stoler, 2009).

Detecting known genetic mutations

PCR is very effective in detecting known genetic mutations such as substitutions, deletions and insertions. These processes are found in many oncologic diseases and identifying these alterations allows the diagnostician to confirm the diagnosis, identify carriers of the genetic mutation, provide prognostic information, monitor for disease recurrence and help in selecting appropriate treatment regimes. This last example can be illustrated by the advances in treating patients with lung cancer who carry specific epidermal growth factor receptor (EGFR) mutations (Tubbs and Stoler, 2009).

Detecting chromosomal rearrangements

Rearrangements include translocations, deletions, inversions and insertions. If the breakpoints between the two genes are known, primers can be used that span across the breakpoint, amplifying it. Follicular cell lymphomas are an example of a disease characterised by a translocation, the commonest translocation in lymphoid malignancies. Bcl2 is an anti-apoptosis protein which allows germinal centre B cells to survive. Normal germinal centres in lymphoid follicles have some B cells undergoing apoptosis. In follicular lymphoma, apoptosis is halted due to the overproduction of bcl2. This overproduction is secondary to a translocation between chromosome 14 and chromosome 18 that places the bcl2 gene next to the immunoglobulin heavy chain locus gene enhancer resulting in unopposed bcl2 production. (Kumar et al, 2010)

Microsatellite amplification

Hereditary nonpolyposis colon cancer (HNPCC) is an autosomal dominant condition caused by inactivation of mismatch repair genes. Microsatellites are 1-9 base pair sequences that are repeated several times. These are prone to errors during replication and in such cases, corrected by the DNA mismatch repair system. Defects in these genes lead to microsatellite instability which can lead to adenocarcinoma of the colon (Kumar et al, 2010). There are at least five mismatch repair genes but the two most commonly implicated are MutS homologue 2 (MSH2) and MutL homologue 1

(MLH1). Germline mutations in these two genes resulting in loss of expression accounts for 80-90% of HNPCC cases. Epigenetic silencing, involving methylation of the MLH1 promoter is responsible for sporadic cases. PCR can be used to screen individuals with these genetic defects, aiding in the management of these patients. (Tubbs and Stoler, 2009).

1.5   RT-qPCR as an alternative to FS and TIC

An alternative to frozen section and touch imprint cytology is the application of quantitative real- time reverse transcriptase polymerase chain reaction (RT-qPCR) in an intra-operative assay to detect the presence of markers of metastatic spread in the lymph node. It fulfils the requirements needed for a fast, specific, sensitive and cheap assay.

1.6   DNA replication and PCR

PCR is a tool that has revolutionised molecular pathology. It can be thought of as a DNA photocopier, mimicking DNA replication in the nucleus of the cell, allowing exponential amplification of a specific target sequence of DNA, leading to a plentiful supply which can be easily detected.

1.6.1   DNA replication in the cell

DNA replication is a complex process but ultimately relies on the separation of the double-stranded DNA (dsDNA) by the enzyme DNA helicase into two strands, with each strand acting as a template for the formation of a new strand. Deoxyribonucleotide triphosphates are coupled onto the growing strand of complementary DNA by the DNA polymerases. This is achieved by adding a phosphate group onto the 3'-OH group, thus synthesising new DNA molecules in the 5'-3' direction. The leading strand of DNA is synthesised in the 5'-3' direction using a single RNA primer, however, the lagging strand is synthesised in pieces (called Okazaki fragments) as DNA polymerase can only add to the 3' end. RNA primers allow the DNA

polymerase to initiate extension. Primers consist of short (10-20) nucleotides and are synthesised by RNA polymerase. They are later replaced by DNA (Manson et al, 2002).
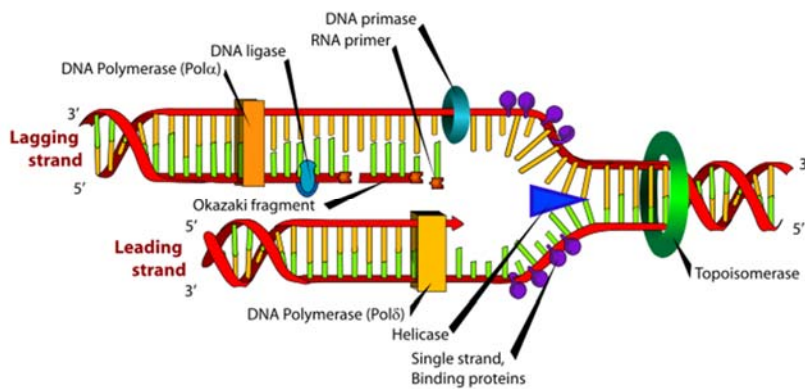
Figure 8. The figure illustrates the process of DNA replication whereby the dsDNA is separated and two complementary strands are produced to form two new dsDNA molecules (About.com, 2011).

1.6.2 PCR steps

PCR aims to replicate the cellular process of DNA replication and therefore requires a DNA template, DNA polymerase and the four deoxyribonucleotide triphosphates (dNTPs) and a forward and reverse primer to target the sequence to be copied. The essential element for successful PCR is thermal cycling. The amplification process is based on repeated cycles of three steps with differing temperatures.

Step 1: Denaturation

Usually held at 95°C, this brief step of up to 30 seconds (s) breaks the hydrogen bonds between the dsDNA, resulting in two single strands of DNA.

Step 2: Annealing

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Temperatures range between 45-60°C and this step allows hybridization or annealing of the forward and reverse primers to their complementary strands.

Step 3: Extension

What follows is the DNA polymerase recognizing and binding to the primers and begins the process of replication by adding complementary dNTPs to the 3' end of the primer strand. The optimal temperature depends on the DNA polymerase used, usually at 72°C. The time taken for this step depends on the length of the target to be amplified (Tubbs and Stoler, 2009).

At the end of the first cycle, two strands are produced, one from each original template strand. These serve as templates for the next cycle and so on, thereby resulting in an exponential amplification of the target. In the early stages of the reaction, the number of amplicons doubles with each cycle and the efficiency of the PCR is 1.0. However, there comes a time when the efficiency plateaus and no more amplicon product is made. This is the result of either a limiting factor in the reaction of one of the reagents or of the instability of the DNA polymerase due to the high number of cycles. Other causes include end product inhibition, competition by nonspecific products, incomplete denaturation of the dsDNA strands at higher levels of product or incomplete reannealing with higher concentration of product (Tubbs and Stoler, 2009).

1st cycle of PCR

Denaturation → 95°C, 15-60 sec.

Annealing → 45-60°C, 30-60 sec.

Extension → 70°C, 1-4 min

Completetion of 1st cycle of PCR

2nd cycle of PCR

3rd cycle of PCR

2 double-stranded DNA molecules

4 double-stranded DNA molecules

8 double-stranded DNA molecules
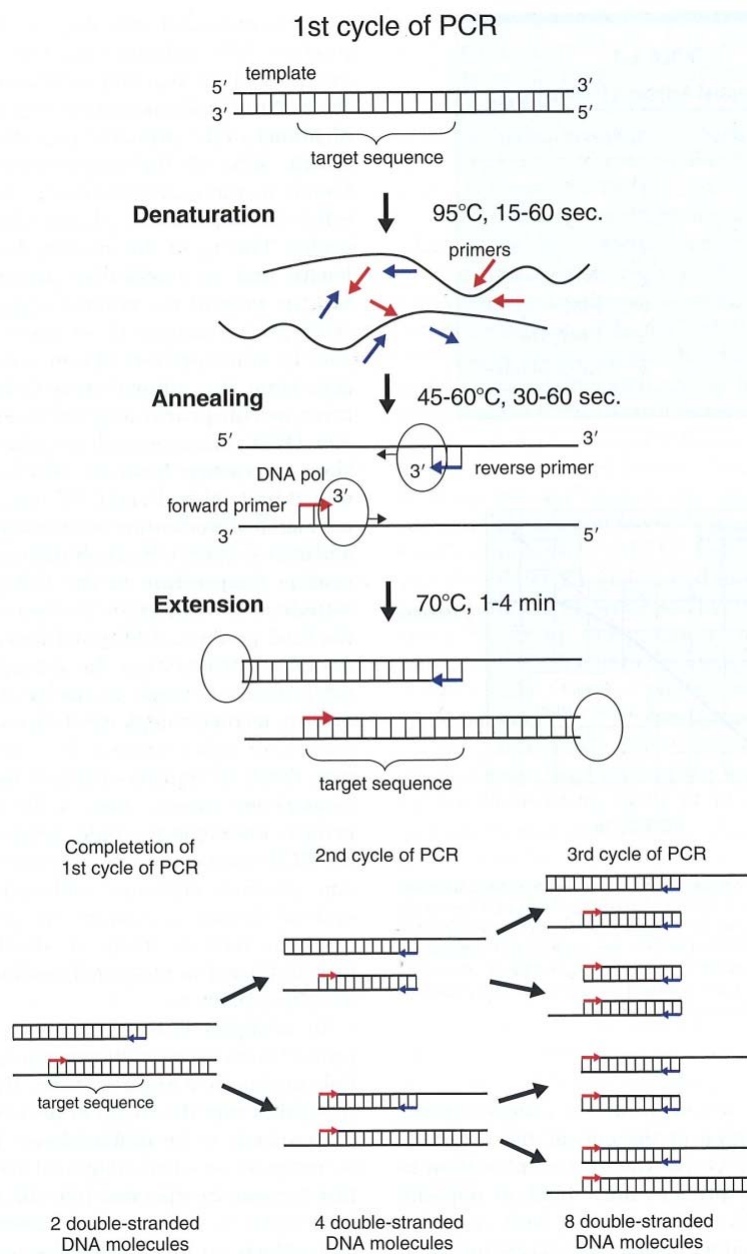
Figure 9. The figure illustrates the three steps in PCR and the exponential amplification of product (Tubbs and Stoler, 2009).

Each component involved in the PCR is discussed.

<u>DNA polymerase</u>

Heat stability is an essential requirement for a DNA polymerase. It was discovered that the DNA polymerase from the bacterium thermos aquaticus, which lives in water with a temperature of 75°C, has an optimum temperature of 72°C and is stable up to 94°C. Taq polymerase is one of the most commonly used polymerases (Tubbs and Stoler, 2009).

<u>The primers</u>

The specificity of the target sequence is guaranteed by primers, short oligonucleotides that contain sequences complimentary to the opposite ends of the target template.

A primer can be defined as an oligonucleotide that initiates polymerase-catalyzed additions of dNTPs by annealing to a template strand (Bruns et al, 2007). There are two primers needed for PCR. The forward primer is complementary to the reverse template strand at the beginning of the target sequence and the reverse primer is complementary to the forward strand at the end of the target sequence. A large number of primers are added to the reaction mix as with each cycle they are incorporated into the new strands, reducing their concentration. Primers are essential in amplifying the correct sequence and they dictate the quality and specificity of the amplification reaction. There are software tools that can select the best primers to use for a specific target. Several properties of a good primer include:

- Primers that are specific to the amplicon. Amplicons are PCR products and are replicated target molecules whose ends are defined by the primers. Primers that even partially match other sequences of the DNA can lead to non-specific PCR product. When designing primers, common repeated sequences should be avoided. Other targets to avoid are pseudogenes. These are genes that, due to accumulated mutations, do not result in a functional gene product. Sequence variations in these pseudogenes are rarely clinically significant.
- Avoiding primers that anneal to themselves or to other primers in a multiplex reaction. This is especially important at the 3' end. Primer dimers can occur

before PCR begins, when at lower temperatures a primer may anneal to another and Taq polymerase may extend it. If it is in turn extended, then double stranded products are formed creating primer-dimers. These can decrease the efficiency of the PCR reaction and reduce the sensitivity of the reaction. Hot start PCR uses an antibody to bind and inactivate the polymerase at room temperature, reducing the chances of primer-dimer formation.

- Avoiding primers with sequences that can be complementary to internal sequences of the amplicon

- Primers with 18-25 bases have a greater chance of being unique in the genome than those with fewer bases.

- Primers that match each other in melting temperature (Tm) so that both anneal at the same temperature. The Tm of the primers is defined as the temperature in which 50% of the oligonucleotide primers are bound to their complementary sequence and 50% separated into single-stranded molecules. The Tm depends on the primers' length ie the number of nucleotides, and their sequence composition ie the number of guanines to cytosines and adenines to thymidines. The Tm can serve as a guide to the optimum annealing temperature, however, in practice this is usually by trial and error.

- In order to minimise amplification of genomic DNA which can lead to false-positive results, the primers can be designed to be exon-spanning (Tubbs and Stoler, 2009).

The template

RNA that is extracted from the tissue sample must be reverse transcribed into cDNA in order to serve as a template in PCR because Taq polymerase cannot transcribe RNA. The enzymes used are from retroviruses that integrate their RNA genomes into host genomic DNA. The reverse transcription (RT) step that precedes the main PCR reaction involves oligonucleotides that anneal to the template and can occur during a single temperature. When RT is combined with PCR it is called a one step RT-PCR reaction. This is where all the reagents needed for both RT and PCR are added to the same reaction mix. Two different enzymes ie DNA polymerase and reverse transcriptase use a DNA polymerase with reverse transcriptase activity eg rTth from the organism

Thermus Thermophilus. When manganese ions are present, reverse transcriptase occurs. The advantage of one-step RT-PCR over two-step PCR, where the reverse transcription is done in a separate reaction tube, is that the risk of contamination is reduced as the tube does not need to be disturbed.

Another component of the PCR mixture is magnesium chloride required for activity by the DNA polymerase. It is bound by the polymerase and affects the Tm of the template, primers and PCR product. Buffer solution is also part of the reaction mixture, keeping the pH of the solution constant for the optimum function of the polymerase (Tubbs and Stoler, 2009).

Once the desired amplicon has been amplified, there needs to be a way to detect the presence and amount of amplicon.

1.7   Real-Time quantitative RT-PCR (RT-qPCR)

Real-time quantitative RT-PCR combines PCR amplification with the detection of the amplicon. It is a tool that allows detection and measurement of products generated during each cycle. The amount of the amplicon produced is directly proportional to the amount of the template. Detection and monitoring of the amplicon can be done by using fluorescently-labelled oligonucleotide probes during amplification of the product ie in real-time. After each PCR cycle, the fluorescence can be measured either during annealing or at the end of the cycle depending on the chemistry used (Arya et al, 2005).

1.7.1   Hydrolysis probes

Hydrolysis probes, also called Taqman probes, are dual-labelled oligonucleotides. They carry a reporter fluorophore at the 5' end and a quencher molecule at the 3' end. The quencher dye absorbs the light energy that is emitted from the reporter dye. When the probe binds to the target sequence during the annealing and extension steps, the DNA polymerase cleaves the 5' end of the probe, releasing the reporter fluorophore.

This results in the emission of light from the reporter dye which is detected by the real-time instrument. The amount of fluorescence is directly proportional to the amount of amplicon produced and the more target there is, the earlier the detection of the fluorescence occurs (Tubbs and Stoler, 2009).
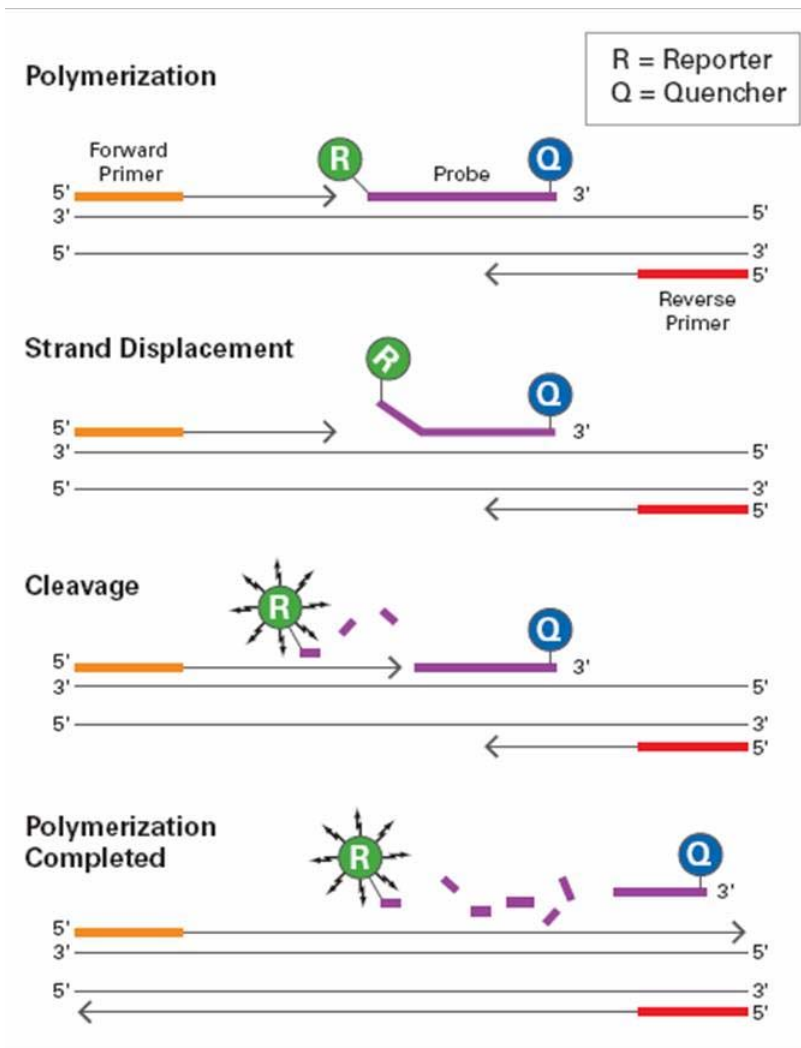
Figure 10. The figures illustrates the sequence of events when the Taqman probe's reporter dye is cleaved, resulting in the emission of fluorescence light which is detected by the instrument (DNA Vision, 2011).

Multiplexing is when more than one marker is used in a single RT-PCR reaction. This is made possible by using different probe sets. However, the more markers that are detected, the more fluorochromes there are in the reaction mixture which might have a detrimental effect on the efficiency of the PCR. It has been recommended not to use more than four markers in a single reaction, otherwise the efficiency would be affected (Tubbs and Stoler, 2009).

1.8   Amplification

The intensity of the fluorescence from a PCR reaction can be plotted against the number of cycles to provide an amplification curve. There are certain properties of the amplification plot including the threshold, baseline, fluorescence emission and cycle threshold (Ct) or quantification cycle (Cq).

The threshold level is in the early stage of the exponential growth phase and is arbitrarily chosen by the computer, being instrument and operator specific. It is calculated as ten times the standard deviation of the average signal of the baseline fluorescent signal between cycles 3 to 15. This threshold can be changed manually in experiments so that it is in the area of exponential amplification.

The baseline is also arbitrarily set by the computer software to be between cycles 3 to 15 and is defined as the cycles where the reporter fluorescent is below the detection level of the instrument during its initial accumulation (Arya et al, 2005).

The Cq value is the number of cycles it takes for a set fluorescence intensity threshold to be reached. It is the cycle number at which the reporter fluorescence is greater than the threshold level which is the minimum detection level. The lower the Cq value, the

higher the amount of initial target and the higher the Cq, the lower the amount of target.

The fluorescence emission is calculated by the computer software by subtracting the fluorescence emission of the baseline by the fluorescence emission of the product at each time point. These values are plotted against the cycle number. During the early stages of the amplification, the values do not exceed the baseline and then suddenly exponentially rises, crossing the threshold level ie the Cq point (Arya et al, 2005).
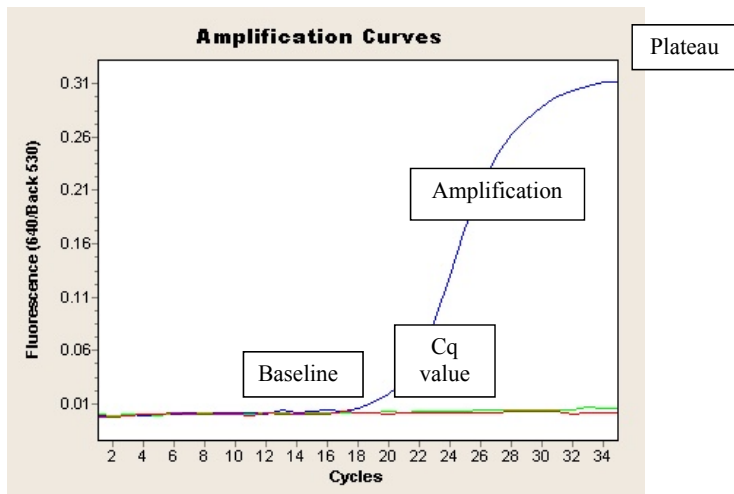
Figure 11. The graph gives an example of a RT-PCR amplification curve from the LightCycler 2 instrument. It plots the fluorescence emission vs the number of cycles.

1.9   Absolute and relative quantification and reference genes

RT-qPCR can be used to quantify the amount of target produced, either by relative quantification or absolute quantification.

Absolute quantification determines the amount of a target (expressed as a copy number or concentration) relative to a standard curve. The standard curve is the plot

of the log of initial target copy number for a set of known standards versus Cq. The standard curve is plotted as a straight line and can be calculated by computer software on RT-PCR instruments. The known target can be either a plasmid of the gene of interest, a single-stranded oligonucleotide for the entire amplicon or a cell line with a known copy number or expression level of the gene (Arya et al, 2005).

Relative quantification determines the ratio between the amount of a target and the amount of a reference nucleic acid, usually a suitable reference gene (Arya et al, 2005). Mathematical equations are used to calculate the expression levels of the target gene relative to the reference control. The reference control could be RNA from normal tissue. The amount of gene target in the sample can be calculated by subtracting the Cq value of the reference gene from the Cq value of the target gene. In order for this method to be valid, the amplification efficiencies of both reference and target genes should be approximately equal. This can be determined if the Cq values of both genes vary with template dilution (Arya et al, 2005).

Housekeeping genes, or reference genes, are genes that are involved in the basic functions needed for the cell to survive and are usually always expressed ie always turned on (Tubbs and Stoler, 2009). They lend themselves to act as internal controls in PCR reactions. Their expression should be constant across all tissues, in all stages of development and in different experiments. In PCR reactions, it can be used to normalize the reaction. In order to correct sample-to-sample variation caused by RNA volume differences, variable RNA quality or differences in efficiency of cDNA synthesis, the reference gene can be amplified along with the target gene. It serves as an internal reference whereby the RNA values can be normalised against. Examples of reference genes include glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β actin. The reference gene used as an internal control in the Metasin diagnostic assay is porphobilinogen deaminase (PBGD) as it is known to be present in every cell type. It is thought that not one unequivocal reference gene has been found and that therefore, using several genes and their mean expression is best practice (Arya et al, 2005).

1.10   Speed is an important factor of intra-operative real time RT-qPCR

RT-qPCR lends itself well to functioning as an intra-operative test. As well as accuracy of diagnosis and cost of the assay, speed is important. Surgeons will want an answer as soon as possible and anaesthetists will not want to delay the operation longer than necessary. As time is taken in transporting the material to be tested, every aspect of the test should be scrutinised to see if it could be speeded up. There are several ways in which to speed up a PCR reaction including using silicon columns and vacuum pressure for nucleic acid purification, changing the denaturation and extension times and reducing the ramp time by lowering the temperature used in the denaturation step for amplicons less than 100 base pairs (McPherson and Moller, 2006).

The fastest method of purifying the nucleic acids after extraction is through applying a vacuum pressure and running the homogenate containing the nucleic acids through a column containing either silica, cellulose or other material. With a high salt binding buffer, the nucleic acids stick to the column and can be washed with a high salt buffer to remove any lysis buffer. It can then be eluted using a low salt solution or water.

DNA polymerase enzymes and reverse transcriptases can add 20 to 100 bases per second, allowing the target RNA to be copied into cDNA in seconds. The PCR product can be replicated with short extension times. To speed up the PCR reaction, the time taken for extension can be reduced, especially for amplicons less than 120 base pairs, as well as the time taken for reverse transcription. Caution should be used not to jeopardise the sensitivity of the assay (McPherson and Moller, 2006).

By using a PCR machine that can heat and cool the PCR mix quickly, ramping times between temperatures can be reduced. One of the fastest PCR instruments is the SmartCycler. Ramping times can also be reduced by lowering the temperature changes needed during cycling. Depending on the number of base pairs the amplicon has, the temperature can be lowered. However, during the first few cycles, the PCR target is cDNA which denatures at a high temperature, usually around 95°C. Therefore, the denaturation temperature can be dropped after the initial cycles (McPherson and Moller, 2006).

Other ways of reducing the time taken to run an intra-operative assay is to allow for analysis of the result during real-time so that once a result becomes obviously positive or negative, the user does not have to wait till the last cycle to communicate the result to waiting surgeons. By training laboratory staff to carry out the procedure, the assay can be run faster (McPherson and Moller, 2006).

Ways of optimising PCR

A reaction buffer containing potassium chloride and magnesium chloride is usually supplied by the manufacturer and the concentration of magnesium ions can be changed to optimise the reaction. The efficiency of the primers is important and their concentration can also be changed, as well as the concentration of the probes. The volume of template can be increased or decreased to try and improve the results (McPherson and Moller, 2006).

1.11 Advantages of an intra-operative RT-PCR assay

There are clinical situations in which an intra-operative diagnosis is required. As discussed earlier, morphological techniques such as frozen section and touch imprint cytology are used in such situations. However, these methods have variable sensitivity and specificity. An advantage of PCR assays is the high level of sensitivity, where theoretically only a few cells with the target RNA need to be present for the PCR assay to detect them. The morphology based tests can only allow representative examination of 2 dimensional structures of the tissue whereas PCR allows the whole tissue to be examined, reducing the chances of a tumour being missed. The results obtained from PCR assays are usually more objective, ie the target is either present or not, whereas histopathological assessment in difficult cases can be subjective. This may be even more marked in departments where there are inexperienced histopathologists reviewing the slides. The PCR assay also reduces the need for a Consultant Histopathologist to be on-call when the tissue comes in as the assay can be carried out by the biomedical scientist.

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1.12 Disadvantages of an intra-operative RT-qPCR assay

The specificity of the PCR assay depends on the choice of the target amplicon. Ideally, the amplicon needs to be specific for the particular tumour being tested for. Unfortunately, for most tumours there is not yet a universally accepted marker. The PCR assay may be too sensitive at times, picking up background expression of the marker being targeted that may have no clinical significance, for example detecting cytokeratin-19 (CK19) in lymph nodes with isolated tumour cells which have no clinical significance. A quantitative assay would help to measure the level of expression and distinguish it from the background expression. Another potential problem with PCR for some Histopathologists is the absence of a morphological picture to accompany the PCR diagnosis. With no histology to validate the assay test, the PCR test would have to be trusted. Ways of getting around this is to take part of the tissue for histopathological assessment and the other part for the assay. However, this could lead to sampling errors if the desired target is in only one part of the tissue. Contamination is also a problematic issue if it is introduced into the reaction. The source of the contamination could be due to neighbouring tissue being introduced to the tissue being tested, using the same cutting instrument between different tissues and not swapping gloves between handling different tissues and preparing the PCR mixture, as well as other ways of contamination. The assay would not be able to differentiate between contamination and a true result. Other issues such as cost may be a factor in some situations, especially if the assay only supplements and does not replace other intra-operative diagnostic tests (Tubbs and Stoler, 2009).

1.13 Markers for detecting metastatic breast cancer in lymph nodes

One of the most important aspects of a PCR assay is the choice of marker and the design of the primers. A suitable marker for metastatic breast cancer should not be present in normal lymphoid tissue and should also be present in breast cancer that has metastasised. As RT-qPCR is becoming widely accepted as an intra-operative diagnostic tool, there have been numerous studies that have searched for and tested suitable markers.

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1.13.1 Technical approaches to identifying overexpression of genes

There are several tools used in identifying genes that are overexpressed in certain tumours. If a gene was found to be expressed in a tumour or normal tissue of interest, then it could potentially become a marker for the tumour or normal tissue in a RT-qPCR assay. One of these tools, the microarray, was described in section 1.4.1. However, there are certain drawbacks to this method of identifying candidate genes. It relies on hybridization of the sample mRNA to oligonucleotides or DNA fragments. These must be in greater excess than the mRNA of the sample. The more genes the microarray has, the more difficult it is for all the mRNA to hybridize, leading to a false impression of its quantity. Cross-hybridization is also a problem if two mRNAs contain similar sequences and they bind to the same probe. Another method of identifying genes of interest and that avoids the problem of microarrays is DNA sequencing (Watson, 2007). Sequencing is any method that determines the order of bases in a DNA fragment (Bruns et al, 2007). The Sanger method is the commonest method of DNA sequencing. It involves a PCR reaction to produce complimentary strands (cDNA) of the target area using primers. Four PCR reaction mixes are prepared, each containing regular dNTPs and one of four dideoxynucleoside triphosphate (ddNTP), a chain terminating nucleotide. The ddNTPs lack a 3'OH group needed to form a phosphodiester bond between two nucleotides, thereby terminating the DNA extension. Extension starts at the same location but finishes at different locations. A low concentration of ddNTP results in fragments of DNA up to 1000 base pairs whereas reaction mixes with a higher concentration of ddNTP would result in shorter DNA fragments. At the end of the PCR reaction, different length strands of DNA are present, all terminating in the ddNTP that was present in the reaction mix. Determining the sequence of a large segment of DNA involves generating many short sequence reads from overlapping sections of DNA. A gel is then used with each of the four reactions run in one lane where the DNA fragments are separated by size. The DNA sequence can then be directly read off the gel or analysed by a computer. By sequencing part of the genome of a breast cancer tumour cell, new genes can be identified which could possibly be candidates as molecular markers. cDNA libraries are freely available to any

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researcher wishing to use the genetic sequence of a target of interest. Expressed sequence tags (ESTs) are small (200-500bp) sequences of DNA generated by either one or both ends of an expressed gene. mRNA is reverse transcribed into cDNA. As the mRNA contains only the exons of genes that are being expressed by the cell, it is the cDNA that is sequenced. The result are 'tags' of DNA sequences that are expressed by the target which can be stored in publically accessible databases. Researchers can use the frequency of ESTs for each gene as an estimate of the expression level of that gene. The limitation with using EST databases is that the researcher is limited to the tissues and cells that were analysed previously in EST projects. Despite this, ESTs have been invaluable in helping to map the human genome and identifying new gene targets in hereditary diseases (National Centre for Biotechnology Information, 2010). Finally, another sequence-based technique, serial analysis of gene expression (SAGE) allows the study of gene expression in a variety of cells. Like microarray analysis, SAGE is a method of quantitatively measuring the expression levels of all transcripts expressed by a cell. In tumour cells, this would highlight all the genes expressed by them. Where SAGE differs from microarrays, is that there is no hybridization involved and therefore no risk of cross-hybridization. It also allows new genes to be discovered (National Centre for Biotechnology Information, 2010). There are several main steps. First, the mRNA of the cell of interest are bound to beads containing oligo(dT) and reverse transcribed into cDNA. Then the cDNA is cleaved by a restriction enzyme, leaving the 3' end attached to the bead. Another restriction enzyme is applied which recognises a particular site and cleaves a few bases from it. This releases the tags from the sequence which can then be concatenated ie cloned into a vector plasmid, and finally sequenced. The abundance of each tag is quantitated allowing the mRNA abundance to be known. The genomes of normal and cancerous cells can be compared (Watson et al, 2007). In one instance where SAGE has been used in breast cancer, four different tumour progression stages were studied (Porter et al, 2001) where it was found that the most consistent and dramatic changes in gene expression was the point where normal mammary epithelium changes to ductal carcinoma-in-situ (DCIS). The genes identified in this transition may become markers for targeted therapy.

1.13.2 Markers identified for breast cancer

Various studies have been carried out to try and identify optimum markers for detecting breast cancer in a tissue using PCR (Min et al, 1998 and Bostick et al, 1998). One such study was carried out by Backus et al (2005) in conjunction with Veridex, the makers of the GeneSearch breast lymph node assay. In this study, the investigators used microarray analysis, EST libraries and primary literature searches to identify candidate markers for diagnosing the presence of metastatic breast cancer cells in lymph nodes. EST libraries for normal breast tissue, breast tumour and peripheral blood were searched providing several hundred thousand genes. These were whittled down by ruling out breast genes that were also found in peripheral blood and those that had a low expression in breast tissue or breast tumour. The breast ESTs were then compared to ESTs found in colon, lung and ovarian libraries and if any matched, they were eliminated. A literature search was then conducted to see if any of the markers were known or thought to be breast tissue or breast tumour specific and given a higher priority. Microarray analysis was then employed and the expression levels of the probe sets were measured in the RNA samples of various tissues including breast, colon, lung, ovary, prostate and blood. The best candidates with the highest expression in breast tissue were selected for further testing using RT-PCR on various tissues. Those that passed this initial PCR test were then validated using sentinel lymph node tissue. Seven markers in all were identified out of the initial several hundred thousand candidate markers. They included Mammaglobin (MGB), CK19, PDEF, PIP, B305D, GABA and B726. The investigators wanted to identify two or more genes that were the most sensitive and specific for metastatic breast cancer. After further testing using RT-qPCR, it was found that the optimum markers were a combination of CK19 and MGB when compared to histology, initially with 90% sensitivity and 94% specificity. It had a positive predictive value (PPV) of 85% and a negative predictive value (NPV) of 96%. At least three genes were found to be significantly expressed in 6% (11 out of 183) of pathology-negative patients and a specificity of 94% was chosen for assessing the sensitivity of individual and combination markers.

Out of 254 sentinel lymph nodes tested, 64 were found to be positive for both CK19 and MGB and histopathological assessment. 172 were found to be negative by both.

Regarding the discordant samples, 11 were negative for histology and positive for CK19/MGB and 7 were positive on histology and negative for CK19/MGB. The investigators describe 5 of the 7 'false-negative' samples as having micrometastases on histology, attributing sampling error for the discordance. They also believed the 11 'false-positives' were metastases missed on histology. They arrived at this conclusion by comparing the expression of CK19/MGB of these samples to known positive nodes and found the level of expression was comparable. They were also positive for the other markers being tested. Further histopathological examination of one of these samples found the node did contain metastases. On further testing of these discordant samples, it was found that the sensitivity of CK19/MGB was 91%, the specificity 97%, PPV 93% and NPV 96%. Using statistical analysis, the cut-offs were determined for MGB at Cq 31.7 and CK19 at 30.9. In summary, the study identified the markers CK19 and MGB (90% sensitivity and 94% specificity) as the optimum combination to detect metastatic breast cancer from several hundred thousand potential markers by employing a genome-wide search using several filters. Previous studies have also found these markers to be potential candidates for diagnosing metastatic breast cancer (Gillanders et al, 2004, Mitas et al, 2001, Manzotti et al, 2001 and Brown et al, 2006).

Based on the findings of the study by Backus et al (2005), CK19 and MGB were chosen to be the markers for the Veridex GeneSearch assay, with PBGD chosen as the reference gene.

1.13.3 Cytokeratin 19 (CK19)

Cytokeratins are intermediate filament proteins which are components of the cytoskeleton of all epithelial and some non-epithelial cells, providing mechanical support. There are two types of cytokeratins, basic type 2 (cytokeratins 1-8) and acidic type 1 (cytokeratins 9-23). Each type 1 pairs with a type 2 cytokeratin so that all epithelial cells have two different types of cytokeratin, except CK-19 which does not pair with another cytokeratin. They can also be classified according to their size as high or low molecular weight cytokeratins. CK-19 is the lowest molecular weight

cytokeratin. There are twenty different types of cytokeratin and they are used in the diagnostic work-up of tumours, aiding in identifying carcinomas. CK-19 is found in a variety of tissues including glandular-type epithelium, such as breast and colon. They are useful in confirming whether a metastatic tumour is of epithelial origin (LaCroix, 2006). In lobular carcinoma, some histopathologists use a pancytokeratin marker to identify metastatic tumour cells which can be difficult to pick up from the background lymphoid cells.

False-positivity has been known to be a problem when using CK19 in RT-PCR. Four sources of potential false positivity are:

Illegitimate transcription

Small amounts of mRNA may be expressed by genes that have no physiological role in the cell or tissue. It is thought that every promoter can be activated by ubiquitous transcription factors with an estimate of one tumour marker expressed in 500-1000 non-tumour cells (Zieglschmid et al, 2005). PCR is very sensitive, theoretically being able to amplify one transcript in a single cell and so it would be beneficial to have a Cq cut off to help decide whether a tissue is positive or negative for ck19. A breast-specific marker, such as Mammaglobin (MGB), with CK19 in an RT-PCR assay can help in providing more specificity.

Haematological disorders

When using CK19 in testing peripheral blood for disseminated tumour cells, its expression can be induced by cytokines and growth factors. These are found to circulate at higher levels in inflammatory conditions leading to a higher risk of false-positive results (Ring et al, 2004).

Pseudogenes

These are defined as a gene that does not result in a functional gene product, usually as a result of accumulated sequence changes (Bruns et al, 2007). Two pseudogenes for CK19, CK19a and CK19b, have been described as having significant sequence homology to CK19 mRNA. Therefore, it is important to design the primers well in order to avoid

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amplifying pseudogenes leading to a false-positive result (Savtchenko et al, 1988 and Ruud et al, 1999).

<u>Contamination</u>

This is more likely to occur when testing peripheral blood and occurs when epithelial cells, for example from skin, could be introduced when taking the sample. This error could be minimised by discarding the first sample and testing the second sample of blood (Lacroix, 2006).

CK19 has been shown to be more sensitive in detecting metastases in lymph nodes than routine histopathological assessment (Noguchi, 1996). There have been many studies that use CK19 in a multigene panel and most have shown that CK19 should be included in any assay used to detect breast cancer metastases in lymph nodes (Lacroix, 2006).

1.13.4 Mammaglobin (MGB) (Secretoglobin family 2A, member 1, SCGB2A2)

MGB is a member of the secretoglobin superfamily which also includes uteroglobin (Clara cell protein), lymphoglobin and the lipophilins A, B (BU101) and C (lacryglobin, mammaglobin B). In breast tissue, MGB is secreted as a glycosylated peptide which is covalently associated with lipophilin B. Its function in normal breast tissue is unknown, as is its aetiology in breast cancer (Sjodin, 2003).

This marker was discovered by Watson and Fleming and was claimed to be breast tissue specific, confined only to the mammary glands. Its expression was also found to be independent of tumour stage, grade or histology (Watson and Fleming, 1996, 1999). However, MGB has since been found to be expressed in other tissues including sweat glands in skin, normal uterus, salivary glands, kidney, testis, cervix and rarely in other tissues such as thyroid and ovarian tissue (Lehrer et al, 1998, Carter et al, 2002, O'Brien et al, 2002 and Zehentner et al, 2002). One study found MGB to be present in gynaecological tissues including cervix, uterus and ovary (Zafrakas et al, 2006). MGB was also found to be present in only 23% of 35 breast tumour samples (Watson and

Fleming, 1996). This could be explained by the finding by O'Brien et al (2005) that tumours with a high grade (grade 3) lacked MGB expression. Despite this, MGB has been reported to be the most accurate, breast-specific marker by several studies (Lacroix, 2006). Gillanders et al (2004) tested MGB, MUC1, CEA, PSE, CK19 and PIP for possible candidates in an RT-PCR assay for detecting breast cancer and found MGB to be the most informative marker from the panel. It was the most sensitive marker, expressed in 114 out of 126 (90.5%) patients found positive by histopathological assessment and molecular analysis. They also concluded that the best panel of markers would include MGB, CEA, PIP and CK19. However, multiplexing four markers would be difficult to achieve without adversely affecting the PCR efficiency (Gillanders et al, 2004). Another smaller study found MGB expression to be positive in all 13 histology-positive lymph nodes and negative in 7 histology-negative nodes (Leygue, 1999).

1.14 GeneSearch assay by Veridex

For the intra-operative diagnosis of metastatic breast cancer in sentinel lymph nodes, the GeneSearch assay by Veridex was developed, trialled in several pilot sites and implemented until 2009 when it was withdrawn due to financial reasons. It was a highly successful assay, with high sensitivity and specificity (Blumencraz et al, 2007, Mansel, 2008 and Martin Martinez, 2008).

The GeneSearch assay by Veridex is a RT-qPCR intra-operative molecular diagnostic tool used to confirm the presence or absence of metastatic breast cancer in sentinel lymph nodes. It does this by detecting the expression of the two aberrant genes, cytokeratin-19 (CK19) and mammaglobin (MGB), in the sentinel lymph node tissue. Normally, these two genes should not be expressed in lymph node tissue and their detection confirms the presence of metastatic breast cancer. PBGD is used as the reference gene. A result is expected in less than 45 minutes. The cut off values for whether a tissue sample is valid ie detects PBGD, and whether it is positive for either or both CK19 and MGB is defined by the assay as:

MGB positive for a Cq value less than or equal to 31

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CK19 positive for a Cq value less than or equal to 30

PBGD positive for a Cq value less than 36

There have been several studies comparing the accuracy of the GeneSearch assay with histopathological assessment. The findings of each study, including the overall sensitivity and specificity of the assay, is summarised in the table below.

Study group	Number of nodes (patients)	Positive nodes*(% of total)	Negative nodes * (% of total)	Number of discordants (% of total)	Sensitivity % (patient basis)	Specificity % (patient basis)
Viale et al, 2008	293 (293)	56 (19.1)	210 (71.7)	27 (9.2)	77.8	95
Mansel et al, 2008	124 (82)	8 (6.5)	110 (88.7)	6 (4.8)	88.9	94.6
Martinez et al, 2008	123 (78)	12** (15.4)	63**(80.8)	3** (3.9)	92	97
Tafe et al, 2009	72 (59)	13 (18)	54 (75)	5 (6.9)	88.9	93.5
Funasako et al, 2010	196 (117)	26 (13.3)	157 (80.1)	13 (6.6)	85.1	Not given
Yan-Hui Liu et al, 2010	158 (97)	26** (26.9)	63**(65)	8** (8.2)	83.9	95.5
Somasundar am et al, 2011	266 (166)	47 (17.6)	205 (77.1)	14 (5.3)	100	87.1
Cutress et al, 2010	467 (256)	69** (26.9)	175** (68.3)	12** (4.7)	96	95
Veys et al, 2009	(250)**	37** (14.8)	195**(78)	18** (7.2)	94	93

Table 1. A summary of each study including their sensitivity and specificity.

*Both histological assessment and molecular assessment were in concordance

Detection of ITC in either assay is considered to be negative as it has no clinical significance

** These are the number of patients in the study as the node number was not given

Some studies have given their results either by counting the nodes or the patients. In those studies where the number of nodes were given, the overall sensitivity and specificity of the GeneSearch assay is 87.8% and 92% respectively (total number of nodes = 951). For those studies that have given the number of patients only, the overall sensitivity and specificity of the assay is 93.1% and 94.3% respectively (total number of patients = 681).

The reason for a higher sensitivity and specificity of the assay when on a patient basis is that some patients may have two nodes of which both are positive for metastasis on histology. However, the assay may have only picked up one node. Therefore, on a patient count, the result is positive, however, one node was missed leading to a lower sensitivity on a node basis.

The results, when compared to the overall sensitivities and specificities to conventional intra-operative methods, show a higher sensitivity and a slightly lower specificity.

The table below shows a more detailed analysis of the discordants found in the studies.

Study group	Histology positive (macro)/GS negative	Histology positive (micro)/GS negative	Histology negative/GS positive	Total number of discordant nodes (% of total nodes)
Viale et al, 2008	1	15	11	27 (9.2)
Mansel et al, 2008	0	1	5	6 (4.8)
Martinez et al, 2008	0	1**	2**	3** (2.4)
Tafe et al, 2009	1	0	4	5 (6.9)
Funasako et al, 2010	1	2	10	13 (6.6)
Yan-Hui Liu et al, 2010	0	5	3	8** (5.1)
Somasundaram et al, 2011	1	2	11	14 (5.3)
Cutress et al, 2010	0	2	10	12** (2.7)
Veys et al, 2009	1	4	13	18** (7.1)

Table 2. A summary of the discordants of each study.

GS = GeneSearch assay

** These are the number of patients in the study as the node number was not given

The table shows that macrometastases are rarely missed, with four studies out of nine showing no discordance and five having either one case or one node with macrometastasis missed. Micrometastases were more commonly missed and all the studies attribute the most likely cause as sampling error where the metastasis was only in the slices given to histology. Micrometastases are by definition between 0.2mm and 2mm and as the node is sliced into 2mm slices, it is reasonable to assume that a whole focus could be in only one slice which could be given to histology. In practice, it is difficult to accurately slice a fresh lymph node into 2mm and consequently larger slices may be cut.

For those cases or nodes which were found positive with GeneSearch and negative on histology, there is no way of confirming it histologically, however, the most likely conclusion to reach is again of sampling error, especially if the Cq value of the discordant node is high, indicating a micrometastasis. One hundred percent concordance is not possible due to the nature of sampling the tissue between the two tests. A certain discordance rate is inevitable. To investigate further GeneSearch assay-positive and histology-negative nodes, serial sectioning and IHC can be performed to determine whether there are any metastases, vindicating the GeneSearch assay result. Several investigators did this and found between 1-3 lymph nodes that were previously negative on histology to be converted to node positive. In cases where a macrometastasis is found on histology and is negative on the with the GeneSearch assay, the investigator should be prompted to repeat the assay to rule out any human error. It is less likely that a sampling error could occur unless the deposit is just larger than 2mm eg 2.1mm, or the slices were cut particularly thick at cut-up. Overall, the conclusion of each of these studies was that the GeneSearch assay was a suitable tool for the intra-operative detection of metastatic breast cancer, with the sensitivity better than conventional intra-operative techniques.

1.15   Conclusion

In summary, molecular pathology is the future of medicine, allowing patient-centred, targeted therapy, accurate diagnostic tools, tumour subtyping and prognostic information to be possible and improved on. By understanding the biology of the tumour and its associated genetic alterations and mutations, dozens of advances can be made in diagnosing, treating and monitoring cancer patients. Breast cancer is no exception, with advances in tumour subtyping, prognostic information and diagnosis of tumour spread to lymph nodes bringing in molecular pathology at a practical level.

Accurate intra-operative assessment of sentinel lymph nodes was regarded as a valuable advancement in patient care, avoiding second operations for patients found to have a positive node. Thus, the withdrawal of GeneSearch left a gap in the service.

1.16 Aim of the project

The aim of this project was to develop and validate an alternative method for sentinel lymph node analysis. This assay has been named Metasin.

The specific objectives are:
1. Extracting RNA from homogenates
2. Setting up a PCR reaction mixture with suitable primers and probes
3. Optimising the RT-qPCR reaction
4. Validating the assay and comparing the results to the GeneSearch assay

PART II. MATERIALS AND METHOD

Chapter 2

Unless otherwise stated, all practical work was largely carried out by myself with guidance and support from Dr Sai-Giridhar and Mrs Mascall.

This chapter describes the methods employed in setting up the Metasin assay. A detailed inventory of the materials is provided in the appendix pg 189**.** The first step in setting up the assay was the acquisition of suitable primers and probes for the three markers CK19, MGB and PBGD. The first thermocycler instrument used was the LightCycler 480 (LC480) (Roche). The machine is capable of testing up to 100 samples and was chosen to optimise and validate the assay initially. The appropriate recommended Roche RT-PCR kit was used and the initial optimising runs were carried out. This involved changing the primer and probe concentrations and the template volume. Once the Cq values of the samples run on the LC480 matched closely those of the GeneSearch assay, validation began. The most important characteristic of an intra-operative assay is the speed of the assay. Despite attempts to try and speed up the time taken to reach results, the run time could not be reduced to an acceptable time of less than 26 minutes for one node. The faster SmartCycler (Cepheid) thermocycler was chosen to replace the LC480. The assay was then optimised again on the different platform and a faster run time achieved. Once comparable Cq values were obtained, the homogenates of all nodes were run and the results statistically analysed and compared to the GeneSearch results. Cut-off values for negative and positive nodes for all markers were established and the in-house assay, Metasin, was run alongside GeneSearch, eventually replacing GeneSearch and providing a diagnostic service.

2.1   Ethical approval and Patient selection

All patients undergoing sentinel lymph node biopsy for breast cancer during the period 2009 to mid 2010 at Princess Alexandra Hospital were approached to consent to be part of this study. The inclusion criteria were patients undergoing a surgical excision of

a primary operable breast cancer and sentinel lymph node sampling. No exclusion criteria were named.

The study was carried out after approval by Essex 2 Research Ethics Committee (Ethics Approval Reference: 07/H0302/129).

2.2   Primer and Probe design

The National Centre for Biotechnology Information (NCBI) GenBank sequence database (National Centre for Biotechnology Information, 2009) was interrogated to obtain accession numbers for the markers. PBGD accession number NM_000190, CK19 NM_002276 and MGB NM_002411.

To generate suitable primers for our markers CK19, PBGD and MGB the Roche Universal Probe Library (UPL) website was used (Roche, 2009). The suggested program-designed primer sequences are shown in table 3.

Marker	Probe	Forward primer	Reverse primer
PBGD	probe # 26	tgtggtgggaaccagctc	tgttgaggtttccccgaat
CK19	probe # 71	gccactactacacgaccatcc	caaacttggttcggaagtcat
MGB	probe # 71	ctcccagcactgctacgc	tgtggattgattgtcttggaaa

Table 3. Shows the forward and reverse primer sequences for the markers from UPL

The program-designed UPL primers and FAM-labelled probes were used in the initial optimisation process of the Metasin assay on the LC480 platform. During the optimisation process, it was decided that the primers should be multiplexed in order to improve the efficiency of the assay. To multiplex the primers, each one should be labelled with a different fluorochrome. Since we did not have access to the Roche probe sequences, it was decided to design our own probe sets. The UPL primer sequences, along with their marker accession numbers, were sent to TIB MOLBIOL, a company expert in designing and manufacturing quality oligonucleotides. The probes were made based on the amplicons generated by the primers. The sequences of the

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probes and primers designed by TIB MOLBIOL and used in the Metasin assay on the LC480 are shown in table 4.

Marker	Probe	Probe label	Forward primer 5' to 3'	Reverse primer 5' to 3'	Amplicon length
PBGD	ctcctgaactccagatgcggga	cyan 500	tgtggtgggaaccagctc	tgttgaggtttccccgaat	92
CK19	cagccagacgggcattgtcg	LC610	gccactactacacgaccatc	caaacttggttcggaagtcat	128
MGB	ctctggctgccccttattggag	LC670	ctcccagcactgctacgc	ggattgattgtcttggaaa	69

Table 4. Tib MolBiol-manufactured forward and reverse primer combination sequences, probe sequences, dye label and amplicon length.

The primer sequences given by the UPL website and the primer sequences developed by Tib MolBiol differ by a few bases. The primer annealing temperatures ranged from 56.2°C to 57.9°C. The primers were designed to be intron-spanning to avoid amplifying and detecting contaminating genomic DNA.

The full list of the DNA sequence for each gene and the location of the primers is available in the appendix (pg 193).

While optimising the assay on the LC480, the length of time for the RT-PCR was found to be approximately 45 minutes. Despite attempts to try and reduce the run time, the time taken for a result was far too long to be acceptable for the clinicians and surgeons. Therefore, it was decided to switch the assay to the SmartCycler platform in order to improve the speed of the assay. The same primers and probes used on the LC480 were also used for the Metasin assay on the SmartCycler.

2.3 Processing tissue for histology

During the intra-operative setting, the sentinel lymph node or nodes arrive in the lab where they are booked in and given a lab number allowing the sample to be identified. The Consultant Pathologist clears any excess fatty tissue and slices the node into 2mm slices. Alternate slices are then given to the Metasin assay and to histology.
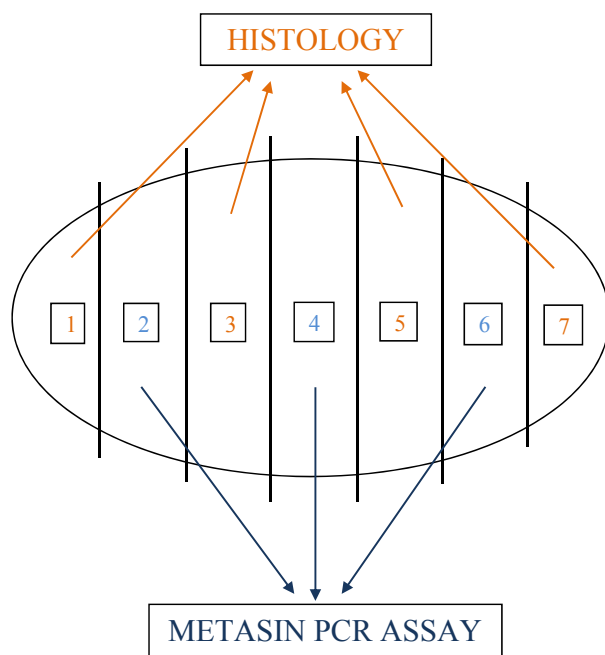
Figure 12. Slicing and allocation of the sentinel node with alternate slices given to histological assessment and the Metasin assay

The slices for histology are placed singly in cassettes and processed per normal procedure. After the tissue is fixed in formalin, it is placed in a tissue processor and washed first in 70% alcohol and then 100% alcohol for five cycles. It is then washed with xylene three times and impregnated with wax. The tissue is then taken to the embedding stage where it is orientated and embedded in wax moulds ready for cutting into thin slices to place on a slide.

Each block is cut to have three levels. The first level is the first full-face of the slice. Then four serials are cut which are kept as spares in case of further work being needed such as Immunohistochemistry or levels. Two more levels are cut and after each level four serials are cut. 150 microns are cut to get the second and third levels and 2 microns for each serial.

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Trim to reach full-face

First full-face = Level 1

2um = serial 1

2um = serial 2

2um = serial 3

2um = serial 4

150um = Level 2

2um = serial 1

2um = serial 2

2um = serial 3

2um = serial 4

150um = Level 3

2um = serial 1

2um = serial 2

2um = serial 3

2um = serial 4

The slides are then heated in an oven, stained with H&E and a coverslip placed.

2.4   The RNA extraction, PCR preparation and optimisation process on the LC480

This section describes the steps taken to extract RNA for use in the assay on the LC480. Steps in preparing the PCR mix for use with cDNA in two-step PCR and RNA in one-step PCR are given as well as the steps taken to prepare the primers and probes for monoplex and multiplex runs. The optimisation process is detailed, including changes in primer, probe and template concentrations and running a dilution series. Finally, attempts to reduce the time of the run protocol are described.

2.4.1   RNA extraction using GeneSearch materials

The RNA used for the assay on the LC480 platform was the same RNA previously extracted from sentinel lymph nodes to be run on the GeneSearch assay. Therefore,

the method of RNA extraction described in this section is the same protocol as that of the GeneSearch Breast Lymph Node (BLN) test kit (Veridex, LLC, Warren, NJ).

On receipt of a new kit, certain preparation of the working reagents are carried out. To prepare working homogenization buffer (contains ≥ 25% guanidine thiocyanate), 1.0mL of β-mercaptoethanol, 14.3M (Calbiochem) is added to one bottle of 100mL homogenization buffer. Working wash buffer 2 is prepared by adding 8mL of absolute 200-proof ethanol (Sigma-Aldrich) to the bottle and mixing well.

The lymph node is sliced into 2mm slices with alternating slices going to the assay for the LC480 and to conventional histology. The lymph node tissue slices allocated to the assay is weighed. The tissue is next diced into small pieces and then added to homogenization buffer. The volume of homogenization buffer to add is dependent on the weight of the tissue. The full table showing the volume of homogenization buffer to be added according to tissue weight is available in the appendix (pg195). 400µl of 70% ethanol (Sigma Aldrich) is added to 400µl of the homogenate and mixed thoroughly by vortexing. A certain volume of the homogenate-ethanol mixture is then transferred to a RNA spin column (GeneSearch) attached to a vacvalve onto a vacuum manifold. The volume of homogenate-ethanol mixture to be transferred to the RNA spin column is dependent on the weight of the tissue. The full table is available in the appendix (pg 196). The vacuum is maintained between 800-1000 mbars until the sample is filtered and the vacuum is turned off. 700µl of wash buffer 1 is added to the column and the vacuum turned on again until the buffer is filtered. 700µl of wash buffer 2 is next added to the spin column and the vacuum turned on again until the mixture is filtered. The columns are then removed and centrifuged for 30 seconds at greater than 10,000RPM in a microcentrifuge. The old centrifuge tube is discarded and the spin column placed in a new collection tube. 50µl of RNase-free water (GeneSearch) is added directly onto the filter membrane of the column. The column is then centrifuged for 30 seconds at greater than 10,000 RPM. The column is discarded and the collection tube contains 50µl of eluted RNA.

2.4.2   RNA to cDNA conversion (2 step RT-PCR)

The steps taken to convert RNA to cDNA are followed in the QuantiTect Reverse Transcription Handbook (Qiagen).

Two mixtures are made for the conversion. The first mixture is involved in eliminating genomic DNA and contains gDNA wipeout buffer. The second mixture performs the reverse transcription step and contains Quantiscript reverse transcriptase (contains RNase inhibitor), Quantiscript RT buffer (contains Magnesium and dNTPs) and RT primer mix.

The vials containing the gDNA wipeout buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix and RNase-free water are thawed, mixed by flicking and spinning and stored on an ice block.

Table 5 shows the volume of each reagent for one reaction

Reagent	Volume/reaction (μl)
gDNA wipeout buffer, x7	2
Template RNA	5
RNase-free water	7
Total reaction volume	14

Table 5: Genomic DNA elimination reaction components for one reaction

The reagents are added to a labelled microfuge tube (Aldrich) and incubated at 42°C using a waterbath for 2 minutes and then put on ice.

The volume of each reagent for the reverse-transcription mastermix is shown in table 6.

Reagent	Volume/Reaction (µl)
Quantiscript reverse transcriptase	1
Quantiscript RT buffer, 5x	4
RT primer mix	1
Template RNA (see table x)	14
Total reaction volume	20

Table 6: Reverse-transcription reaction components for one reaction

The microfuge tube containing the reverse-transcription reaction components is incubated at 42°C for 15 mins and at 95°C for 3 mins to inactivate Quantiscript reverse transcriptase.

The microfuge tube now contains cDNA which can be used on the LC480 or stored in the -20 °C freezer.

2.4.3 Creating the PCR mix for the LC480 platform using cDNA template in two-step RT-PCR

The PCR mix was prepared by following the steps in the LightCycler 480 Probes Master kit insert (Roche). The probes two times master mix vial contains FastStart Taq DNA Polymerase, reaction buffer and dNTP mix (with dUTP) and 6.4 mM MgCl2 (Roche).

Table 7 shows the volume of each reagent for one reaction

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Reagent	Volume/Reaction (µl)
LightCycler 480 Probes Master, 2x conc	10
Primer-Probe mix, 10x conc	2
RNase-free water	3
Total volume	15

Table 7: The volume of each reagent for one reaction using cDNA template on the LC480

The primer-probe mix is prepared by diluting the stock of 100µM of primers and the stock of 10µM of probes with RNase-free water to 10µM and 1µM respectively. 10µl of each primer and probe is added to a single microfuge tube and 10µl of RNase-free water added to bring the total volume to 100µl. The final concentration of each primer is 1.0µM and of each probe is 0.1µM.

The PCR mix is mixed by pipetting up and down and 15µl added to each well of the LightCycler 480 multiwell plate. 5µl of cDNA template is added to each well. The multiwell plate is sealed with LightCycler 480 sealing foil and loaded into the LC480.

The run protocol used on the LC480 when using cDNA is shown in table 8.

Program	Temperature (°C)	Hold (seconds)	Number of cycles
Pre-incubation	95	300	1
Amplification	95	10	45
	60	30	
	72	1	
Cooling	40	10	1

Table 8: The run protocol on the LC480 in two-step RT-PCR

2.4.4 Creating the PCR mix for the LC480 platform using RNA template in one-step RT-PCR

When using RNA on the LightCycler 480, the LightCycler 480 RNA Master Hydrolysis Probes kit (Roche) was used. The LightCycler 480 RNA Master Hydolysis Probes reagent and the Activator reagent are aliquoted into smaller volumes in separate microfuge tubes in order to avoid repeated freezing and thawing. The LightCycler 480 RNA Master Hydrolysis Probes reagent, 2.7 x concentration, contains Tth DNA polymerase, reaction buffer, $MgCl_2$ and dNTP mix with dUTP. The Activator, 50mM, contains $Mn(OAc)_2$.

Table 9 shows the volume of each reagent for one reaction

Reagent	Volume/Reaction (µl)
LightCycler 480 RNA Master Hydrolysis Probes, 2.7x conc	7.4
Primer-Probe mix, 10x conc	2
Activator	1.3
RNase-free water	7.3
Total volume	18

Table 9: The volume of each reagent for one reaction using RNA template on the LC480

The primer-probe mix used in the pilot runs was prepared by diluting the stock of 100µM of primers and the stock of 10µM of probes with RNase-free water to 10µM and 1µM respectively. When performing monoplex runs, 10µl of the forward and reverse FAM-labelled primers and 10µl of the probe of the marker is added to 70µl of RNase-free water to make 100µl containing 1µM of primer and 0.1µM of probe. When multiplexing the three sets of primers and probes designed by TIB MOLBIOL, 10µl of each primer and probe and 10µl of RNase-free water is added to a single microfuge tube to bring the total volume to 100µl. The final concentration of each primer is 1.0µM and for each probe is 0.1µM.

The PCR mix is mixed by pipetting up and down and 18µl added to each well of the LightCycler 480 multiwell plate. 2µl of RNA template is added to each well. The multiwell plate is sealed with LightCycler 480 sealing foil and loaded into the LC480.

The run protocol used on the LC480 when using RNA is shown in table 10.

Program	Temperature (°C)	Hold (seconds)	Number of cycles
Reverse Transcription	63	180	1
Denaturation	95	30	1
Amplification	95	10	45
	60	30	
	72	1	
Cooling	40	10	1

Table 10: The run protocol on the LC480 in two-step RT-PCR

2.4.5 Optimising the assay on the LightCycler 480

The following sections describe the methods used in order to optimise the assay on the LC480, including optimising the primer, probe and template concentrations. Initial pilot runs were performed using monoplexed FAM-labelled primers and later multiplexed primers labelled with different fluorochromes. A dilution series was then run and the standard curve and efficiency calculated by the LC480 software. Finally, an attempt at reducing the run time is described.

2.4.5.1 Changing primer and probe concentrations

RNase-free water is added to the primers and probes to make up stock concentrations of 100μM and 10μM respectively and the vials spun. In order to change the concentrations, the desired volume of the stock is diluted with RNAse-free water. The final PCR mix has a 10X concentration of primers and probes. Primer concentrations of 0.5μM, 0.8μM and 1.0μM and probe concentrations of 0.05μM, 0.08μM, 0.1μM and 0.2μM were tested.

2.4.5.2 Changing template concentration

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Neat RNA was diluted ten fold, 100 fold, 1000 fold and 10,000 fold using RNase-free water and the concentrations tested in a single run.

2.4.5.3 Dilution series and standard curve on the LC480

A 10-fold dilution series of neat, 10 fold, 100 fold, 1000 fold and 10,000 fold cDNA from known positive samples was run. The primer and probe concentrations used were 1.0µM and 0.1µM respectively. The LightCycler software calculated the standard curve and the efficiency.

2.4.5.4 Changes to the run protocol

The original run protocol used for optimising the assay is as follows:

	Temperature (°C)	Number of Cycles	Hold (seconds)
Reverse Transcription	63	1	180
Denaturation	95	1	30
Amplification	95	45	10
	60		*30*
	72		1
Cooling	40	1	10

Table 11. The original run protocol on the LC480

To try and reduce the run time of 52 minutes for 45 cycles, the annealing time of 30s is reduced to 20s and then 10s.
Turbo PCR is then carried out with an annealing time of 30s for 10 cycles, 20s for 10 cycles and 10s for 15 cycles.
A known positive sample is used in these runs.

Despite attempts to reduce the run time on the LC480, the approximate time of 45 minutes was too long for the purposes of providing an intra-operative diagnosis. Therefore, it was decided to switch the assay to the SmartCycler platform as this

promised a faster RT-PCR time. The SmartCycler is also used in the commercial assay GeneSearch BLNA and therefore a quicker run time was already known to be possible.

2.5 The RNA extraction, PCR preparation and optimisation process on the SmartCycler

This section describes the preparation of the PCR mix and the primer-probe mix that was carried out at the time of optimisation and validation. A more streamlined standard operating protocol of the Metasin assay using the SmartCycler is available on page 244.

This section starts by introducing the process of RNA extraction using Qiagen materials, followed by the steps taken for preparing the PCR mix and the primer-probe mix. Finally the optimisation process is described.

2.5.1 RNA extraction using Qiagen materials

The materials used to extract RNA from the sentinel lymph node were taken from the RNeasy Mini kit (Qiagen). The steps taken to extract the RNA were followed using the GeneSearch Breast Lymph Node (BLN) test kit. The method has been described in section 2.4.1 (pg 90). The homogenization buffer is labelled as Buffer RLT in the RNeasy Mini kit. Wash buffer 1 is labelled as Buffer RW1 and Wash buffer 2 is labelled as Buffer RPE in the RNeasy Mini kit.

2.5.2 Creating the PCR mix for the SmartCycler platform

The PCR mix is prepared by following the guidelines in the LightCycler 480 RNA Master Hydrolysis Probes kit insert (Roche).

Table 12 shows the volume of each reagent for one reaction.

Reagent	Volume/Reaction (µl)
LightCycler 480 RNA Master Hydrolysis Probes, 2.7x conc	9.25
Primer-Probe mix, 10x conc	2.5
Activator	1.625
RNase-free water	7.625
Total volume	21

Table 12. Table showing the reagent volumes/reaction

The final volume is 25µl/reaction with 4µl of RNA.

The minimum number of reactions needed for a run is at least 4. These include:

1. Positive control = 1 reaction

2. Negative control = 1 reaction

3. Patient node = 1 reaction

4. *Spare = 1 reaction

*An extra volume of reaction mixture is made to compensate for any pipetting errors.

Before the arrival of the node, a 2.0µl microfuge tube is labelled 'PCR mix' and the vials and primer/probe mix microfuge tubes are thawed on the bench. Depending on the number of nodes expected, the correct amounts of the constituents are added to the microfuge tube. The mixture is mixed well by shaking and the PCR mix microfuge tube is placed in a tray ready to be aliquoted in the reaction tube. The correct number of labelled reaction tubes is placed in a cool box and 21µl of PCR mix aliquoted into each tube.

Next, the positive and negative controls are thawed. The positive control is a known RNA sample that is positive for both CK19 and MGB. The negative control is a known negative RNA sample. 4µl of each control RNA are added to the appropriately labelled reaction tube containing the PCR mix. Once the patient RNA has been eluted, 4µl is added to the appropriately labelled reaction tube containing PCR mix. The reaction tubes are spun for 10 seconds and placed in the SmartCycler machine and the program started.

The results are telephoned through to the surgical team who will either perform an axillary clearance if the sentinel lymph node is positive or take the patient to recovery if the sentinel lymph node is negative.

2.5.3 Creating the primer/probe mix for the SmartCycler platform

Before the RNA is extracted during testing of a sample, the primer and probe mix would have already been prepared, in most cases on the morning of the operation or the day before. The following are the steps taken to prepare the mix.

- A working stock of 100µM of each primer and 10µM of each probe is mixed well by pippetting and placed in a cool box
- A 2ml microfuge tube is labelled with the name 'PBGD/CK19/MGB primer/probe mix' and the date
- For each primer, a 1.5ml microfuge tube is labelled with the primer name and the concentration 2.5µM (the optimum concentration found during the optimisation process). The concentration of 2.5µM is the final concentration in the assay
- The primer-labelled microfuge tubes and the 'PBGD/CK19/MGB primer/probe mix' labelled microfuge tubes are placed in a rack on an icebox
- 15µl of RNAse-free water is added to each primer-labelled microfuge tube
- 5µl of each primer is aliquoted into its respective microfuge tube and mixed well by pipetting making a solution of 25µM of each primer
- Into the microfuge tube labelled 'PBGD/CK19/MGB primer/probe mix' is added:
  - 37µl of RNase-free water
  - 1µl of each probe (3µl in total)
  - 10µl of each primer (3 sets of primers, 60µl in total)

  In total 100µl of primer/probe mix will be made in each microfuge tube with final primer concentrations of 2.5µM and final probe concentrations of 0.1µM
- The aliquot is mixed well by shaking

- The 'PBGD/CK19/MGB primer/probe' microfuge tube is placed in the 4 °C fridge if it is to be used on the same day. If being stored for longer than one day, 25µl of the mixture is added into labelled microfuge tubes and stored in the - 20 °C freezer
- The probe and primer stock are replaced in the - 20 °C freezer
- Details of the fresh batch is documented including the date it was made

2.5.4   Optimising the assay on the SmartCycler

Pilot runs were initially carried out on the SmartCycler using the same optimised variables previously validated on the LC480. The preliminary results were shown to be concordant with GeneSearch with similar Cq values for each marker. The assay run time was reduced by increasing the time of the RT step and by reducing the annealing and denaturing times. The primer concentration and RNA volumes were adjusted to provide optimum Cq values after changing the run protocol. The PCR products were then run on a gel to confirm the identity of the products. The RNA concentrations of a representative group of positive samples were measured on a nanodrop. Monoplexed and multiplexed markers were tested on the same positive samples and their results compared to identify any changes between their Cq values. Finally, a dilution series was run and the efficiency calculated.

2.5.4.1 Reducing the run time on the SmartCycler

The pilot runs were done with the following run parameters

	Temperature (°C)	Hold (seconds)	Number of Cycles
Reverse Transcription	63	180	1
Denaturation	95	30	1
Amplification	95	10	35
	60	30	
	72	1	
Cooling	40	10	1

Table 13. The run protocol used during the optimisation process on the SmartCycler

The threshold for fluorescence for each marker was set to allow for minimum background interference:

PBGD 10.0
CK19 7.0
MGB 4.5

The initial run time was 40 minutes and 12 seconds, which was too long if the assay was to be used as an intra-operative diagnostic assay.
The time had to be shortened without compromising the Cq values. The following changes were done to try and improve run time:

The time taken for the reverse transcription, denaturing and annealing steps were reduced. Turbo PCR was also carried out. The samples used were known positive nodes. The Cq values were then compared to the GeneSearch results of the same samples.

	Temperature (°C)	Hold (seconds)	Number of cycles
Reverse Transcription	63	180	1
Denaturation	95	30	1
Amplification 1	95	1	5
	60	30	
	72	1	
Amplification 2	95	1	5
	60	20	
	72	1	
Amplification 3	95	1	25
	60	10	
	72	1	
Cooling	40	10	1

Table 13.1. The run protocol for turbo 1

	Temperature (°C)	Hold (seconds)	Number of cycles
Reverse Transcription	63	180	1
Denaturation	95	30	1
Amplification 1	95	1	10
	60	30	
	72	1	
Amplification 2	95	1	25
	60	10	
	72	1	
Cooling	40	10	1

Table 13.2: The run protocol for turbo 2

	Temperature (°C)	Hold (seconds)	Number of cycles
Reverse Transcription	63	180	1
Denaturation	95	30	1
Amplification 1	95	1	10
	60	30	
	72	1	
Amplification 2	95	1	25
	60	6	
	72	1	
Cooling	40	10	1

Table 13.3: The run protocol for turbo

Finally, the RT step was changed to 180s, 240s and 300s using the original run protocol.

2.5.4.2 Changing the template volume

RNA volumes of 1-5µl were tested in order to improve the Cq values.

2.5.4.3 Changing the primer concentrations

Primer concentrations of 1.5µM, 2.0µM and 2.5µM were tested in order to optimize the assay even further. Two known positive samples were used. The run protocol recommended by the LightCycler 480 RNA hydrolysis probes kit (Roche) described in section 2.5.4.1 was used. The RNA volume used was 2.5µl.

2.5.4.4 Monoplexed vs multiplexed primers and probes

Six positive nodes were used and on the same run, monoplexes and triplexes were run. The final PCR mixes were run on an agarose gel to ensure the correct products were being detected.

2.5.4.5 Agarose gel electrophoresis of products from the SmartCycler

The loading mixture is prepared by adding 4µl of distilled water, 1µl of 6X blue loading dye (Biolabs) and 1µl of DNA ladder and mixed gently. The mix is loaded onto 1.5% agarose gel (Fluka Analytical). A 1.5% gel was chosen because the higher the percentage of agarose gel, the higher the degree of separation of the lower molecular weight DNA. The gel is prepared using 4.5g of agarose added to 300mls of TRIS borate which has a 10x concentration (30 mls TBA buffer and 300mls water). A hotplate stirrer is used to aid dissolving. 15µl of 0.5ug/µl ethidium bromide is added to the mixture and poured into the tray. ΦX174 HaeIII Digest (Biolabs) yields 11 fragments suitable for use as molecular weight standards.

2.5.4.6 Measuring RNA concentration of GeneSearch and Qiagen extracted RNA

A nanodrop microvolume spectrophotometer (Thermo Scientific) was used to quantify both GeneSearch and Metasin extracted RNA. The purity was assessed using 260/280 ratios.

2.6   Creating the PCR mix for GeneSearch

The GeneSearch BLNA assay is the commercial assay used as the gold standard when comparing the Cq values obtained on the LC480 and SmartCycler platforms.

The GeneSearch RNA Sample Preparation kit (Veridex) provides tubes labelled positive and negative control RNA, a tube labelled 'Mastermix' and a tube labelled 'Enzyme mix'. 10µl from both the Mastemix and Enzyme mix tubes are added to each of the PCR reaction tubes (Cepheid). 5ul of the negative and positive control supplied in the kit is added into the tubes designated as the negative and positive controls and 5µl of the sample to be tested is added in the last reaction tube. The run program on the

SmartCycler is then started. The run protocol used in the commercial GeneSearch BLNA assay is unavailable.

2.7   Statistical analysis

The main objectives to be determined statistically were:
1. Cut-off Cq values for CK19 and MGB for positive and negative nodes
2. Cut-off Cq values for CK19 and MGB for macrometastases and micrometastases
3. The sensitivity and specificity of the assay for the chosen cut-off Cq values
4. The positive and negative predictive value of the assay for the chosen cut-off Cq values
5. The correlation between the two assays using Spearman's rank correlation coefficient

GeneSearch Cq values were taken as the gold standard, and therefore true, for comparing Metasin Cq values of CK19 and MGB. The analysis was done for each node to allow for more data to be evaluated. One of the most important factors was to choose a cut-off that had the least number of false positives and which did not compromise the sensitivity or specificity of the marker.

2.7.1   Determining the cut-off Cq values for positive and negative nodes

When determining the cut-off Cq values for both CK19 and MGB in Metasin, the gold standard was taken to be GeneSearch BLNA assay results. Two different methods were used to arrive at the cut-offs. The first method involved choosing a range of proposed cut-off values for both markers. For each proposed cut-off, the sensitivity and specificity was calculated. This was done for all nodes and also for all cases. The cut-offs chosen were the ones where the results of the maximum number of nodes and cases matched those of GeneSearch. Once the best cut-offs were chosen, their sensitivities, specificities, positive and negative predictive values were calculated and the results were tabulated and compared. The second method involved preparing a scatter plot plotting Metasin Cq values for CK19 vs MGB of all nodes. Each point on the

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scatter plot was also correlated with histology. Vertical and horizontal lines were drawn selecting the best Cq value for both markers which incorporated all the true positive nodes without creating any false-positive node results. Once these cut-offs were established, their sensitivities and specificities were calculated as well as their negative and positive predictive values.

2.7.2 Determining the Cut-off values for macrometastases and micrometastases

All nodes were analysed. Nodes containing isolated tumour cells (ITCs) were considered to be negative. The histology data for each node was used as the gold standard. Using the same scatter plot when choosing positive and negative cut-off values, lines were drawn whereby the best Cq cut-off values were chosen which separated the most macrometastases from micrometastases. The graph is present in section 6.2 (pg 149). The sensitivity, specificity and positive and negative predictive values for the proposed best Cq cut-off values were calculated.

2.7.3 Calculating sensitivity, specificity, negative and positive predictive value

The following were the calculations used to determine the sensitivity, specificity, positive and negative predictive values:

Sensitivity:	True Positive / (True Positive + False Negative)
Specificity:	True Negative / (True Negative + False Positive)
Positive Predictive Value:	True Positive / (True Positive + False Positive)
Negative Predictive Value:	True Negative / (True Negative + False Negative)

2.7.4 The line of agreement

The line of agreement demonstrates the level of agreement between two variables. GeneSearch CK19 and MGB Cq values for all positive nodes were plotted against

Metasin CK19 and MGB Cq values respectively and a 45° line drawn. The line of agreement is where all the points would lie if the two assays were in agreement. The graph provides a gauge of how close the data between the two assays agree. The results are included in section 6.4 (pg 155).

2.7.5 Cohen's kappa

The statistical method of calculating Cohen's kappa using the statistical software SPSS was used in order to show agreement between the Metasin and GeneSearch datasets. The assessment was taken on a patient basis and therefore had a sample size of 154 as the clinical decision is taken on a patient basis rather than on an individual node basis. Cohen's kappa was chosen because it was thought that as there were cases with more than one node, the node results for a patient may not be totally independent of each other as a patient with a macrometastasis could be thought to be more likely to have metastasis in her other nodes compared to those with a negative lymph node.

Chapter 3

This chapter aims to present the clinical information of the participants and to show the results of the optimising steps on the LightCycler 480 and the validation results. Firstly, the best primer combinations were determined using known positive samples. To optimise the assay on the LC480, the primer and probe concentrations were changed to determine the optimum concentrations. The volume of RNA was also changed to see if it would improve Cq values. Once the Cq values were comparable to those of GeneSearch, all the samples were run to validate the assay. No discordants were recorded. Attempts were then made to reduce the run time. However, it became clear that the best time obtained on the LC480 was not suitable for an intra-operative assay.

3.1 Participant demographics, clinical and pathological information

Various clinical and pathological features of the participants were collected and tabulated.

		All Patients	
Patients Enrolled age, years		156	**Total**
mean age at diagnosis	positive SN	60.2	62.2
	negative SN	63.2	
	discordant SN	60.2	
median age at diagnosis	positive SN	59	63
	negative SN	63	
	discordant SN	62	
age range	positive SN	39-92	34-92
	negative SN	34-87	
	discordant SN	43-68	
Surgery Performed			
Mastectomy	positive SN	18	42 (27%)
	negative SN	18	
	discordant SN	6	
Wide local excision	positive SN	18	100 (64%)
	negative SN	82	
	discordant SN	6	
SN/axillary clearance only	positive SN	1	7 (4%)
	negative SN	6	
	discordant SN	0	
Tumour Stage			
pT1	positive SN	18	79 (51%)
	negative SN	61	
	discordant SN	0	
pT2	positive SN	15	46 (29%)
	negative SN	28	
	discordant SN	3	
pT3	positive SN	2	4 (2%)
	negative SN	2	
	discordant SN	0	
Tumour Grade			
Grade 1	positive SN	5	20 (13%)
	negative SN	15	
	discordant SN	0	
Grade 2	positive SN	20	78 (50%)
	negative SN	55	
	discordant SN	3	
Grade 3	positive SN	12	29 (19%)
	negative SN	16	
	discordant SN	1	
Ungradeable	positive SN	0	10 (6%)
	negative SN	9	
	discordant SN	1	

Table 14.1 Patient demographics and clinical and pathological information

Type of tumour			All Patients	Total
IDC	positive SN		26	103 (66%)
	negative SN		74	
	discordant SN		3	
ILC	positive SN		6	16 (10%)
	negative SN		9	
	discordant SN		1	
Others	positive SN		6	32 (20%)
	negative SN		24	
	discordant SN		2	
DCIS	positive SN		0	11 (7%)
	negative SN		10	
	discordant SN		1	
LCIS	positive SN		0	1 (0.6%)
	negative SN		1	
	discordant SN		0	
Hormone receptor status				
ER positive	positive SN		36	130 (83%)
	negative SN		88	
	discordant SN		6	
ER negative	positive SN		1	18 (11%)
	negative SN		17	
	discordant SN		0	
PR positive	positive SN		27	103 (66%)
	negative SN		71	
	discordant SN		5	
PR negative	positive SN		10	45 (29%)
	negative SN		34	
	discordant SN		1	
Her2 positive	positive SN		4	13 (8%)
	negative SN		8	
	discordant SN		1	
Her2 negative	positive SN		33	127 (81%)
	negative SN		90	
	discordant SN		4	
Triple negative	positive SN		1	14 (9%)
	negative SN		13	
	discordant SN		0	

Commented [SP1]: You have not defined cut-off for negative vs positive, as requested previously.

Table 14.2 Patient demographics and clinical and pathological information

*Her2 scoring: immunohistochemistry 3+ score or ISH amplified

*ER and PR scoring: Allred score (positive 3+ or more)

The majority of positive patients included in the practical work have IDC (27cases/37cases) followed by ILC (5/37), with other less common tumours including micropapillary (3/37), tubular (1/37) and mixed IDC and ILC (1/37).

3.2   Results of the optimisation process on the LightCycler 480

Results of the optimisation process including the outcome of primer and probe
concentration changes and changes in RNA volume are given.

3.2.1   Template dilution series, standard curve and efficiency on the LC480

A template dilution series of a positive sample was carried out in order to produce a
standard curve. The efficiency calculated by the LC480 software was 97-98%.
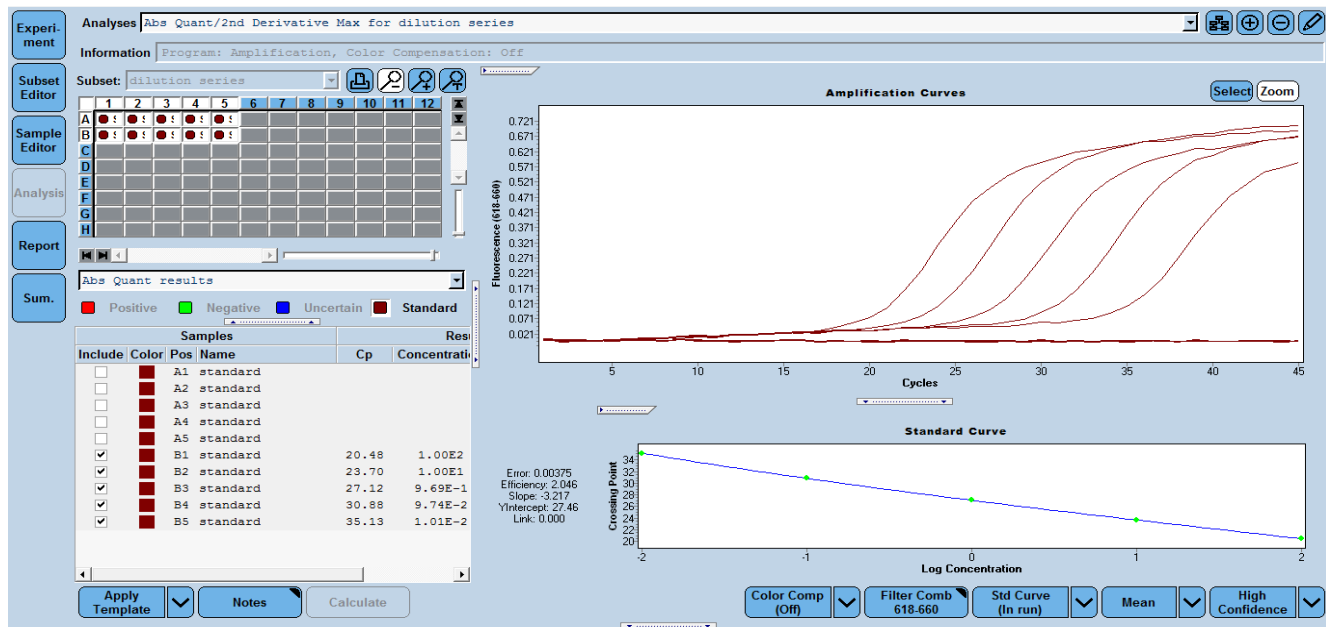
Figure 13. The figure shows the dilution series on the LC480 and the standard curve with an efficiency of 97-98%.

The following tables show the Cq values for the dilutions carried out for each marker.

The GeneSearch Cq value is also provided to compare the Cq values.

Marker	RNA dilution	Cq value	GeneSearch Cq value
PBGD	1	25.76	32.8
	0.1	29.76	
	0.01	32.95	
	0.001	35.91	
	0.0001	ND	

Table 15.1

Marker	RNA dilution	Cq value	GeneSearch Cq value
CK19	1	20.43	19.8
	0.1	23.65	
	0.01	27.12	
	0.001	30.93	
	0.0001	35.12	

Table15.2

Marker	RNA dilution	Cq value	GeneSearch Cq value
MGB	1	21.22	17.1
	0.1	25.96	
	0.01	29.79	
	0.001	33.98	
	0.0001	37.89	

Table 15.3

Tables 15.1 – 15.3 RNA dilution Cq values for each marker compared with GeneSearch

3.2.2 Changing primer concentration

When changing the primer concentrations, the probe concentration was constant at 0.1μM. Table 16.1 shows the different concentrations and their corresponding Cq value for each marker.

Marker	Primer concentration (μM)	Cq value	GeneSearch Cq value
PBGD	0.5	39.13	27.3
	0.8	31.81	
	0.8	31.95	
	1	31.11	
	1	31.24	
CK19	0.5	46.91	18.9
	0.8	18.9	
	0.8	19.09	
	1	18.42	
	1	18.24	
MGB	0.5	24.56	18.3
	0.8	18.25	
	0.8	18.57	
	1	16.79	
	1	17.07	

Table 16.1. Comparing primer concentrations of 0.5 μM, 0.8μM and 1.0μM.

At a concentration of 0.5μM, the PBGD is very high and would have been considered invalid by the GeneSearch assay. There was no significant difference between 0.8μM and 1.0μM. Both concentrations gave Cq values which were found to be comparable to the GeneSearch assay Cq value for the same sample. Therefore, a 1.0μM primer concentration in the assay was used as the calculations would be easier and there was no significant difference between 0.8μM and 1.0μM.

3.2.3 Changing probe concentration

Probe concentrations of 0.05μM, 0.08μM, 0.1μM and 0.2μM were tested to find the optimum concentration. A constant primer concentration of 0.8μM was used. The table shows the Cq values for each marker for a known positive specimen.

Marker	Probe concentration (μM)	Cq value	GeneSearch Cq value
PBGD	0.05	31.70	27.3
	0.08	31.85	
	0.1	31.11	
	0.1	31.24	
	0.2	31.34	
	0.2	31.58	
CK19	0.05	40.52	18.9
	0.08	40.98	
	0.1	18.42	
	0.1	18.24	
	0.2	18.51	
	0.2	19.12	
MGB	0.05	22.27	18.3
	0.08	22.05	
	0.1	16.79	
	0.1	17.07	
	0.2	16.84	
	0.2	17.21	

Table 16.2 Comparing probe concentrations of 0.1 and 0.2μM

When comparing probe concentrations 0.05μM with 0.08μM, there is very little difference between them. There was no significant difference in using 0.1μM or 0.2μM of probe.

The optimum and most economical probe concentration was found to 0.1μM.

3.2.4   Changing RNA volume

The optimum primer and probe concentrations were used when comparing the different RNA volumes. When running three positive samples, it was found that volumes of 1ul, 2µl and 5µl did not significantly change the Cq value. Therefore, a volume of 2µl was used as recommended by the LC480 PCR kit insert. The results can be found in the appendix (pg197).

3.2.5   Results of multiplexing

When the optimum probe and primer concentrations had been found (0.1µM Probe concentration and 1.0µM Primer concentration respectively) they were multiplexed, firstly in pairs and then triplexed. Colour compensation was applied to the runs.
The table shows the Cq Values of the monoplexed and multiplexed markers.

Markers		Cq value
PBGD	PBGD	29.84
MGB	MGB	22.62
CK19	CK19	24.18
PBGD & CK19	PBGD	31.80
	CK19	40.70
PBGD & MGB	PBGD	30.51
	MGB	23.65
CK19 & MGB	CK19	25.59
	MGB	22.83
PBGD&CK19&MGB	PBGD	30.27
	MGB	23.44
	CK19	25.87

Table 17. The table shows the Cq values of monoplexed, duplexed and triplexed markers

When comparing the Cq values of the monoplexed and triplexed markers, the average difference in Cq value is 0.98 suggesting little interference between the nine primer/probe combinations.

The pilot run was encouraging and therefore a batch of known positive nodes were run. Once their Cq values were comparable to GeneSearch, the rest of the positive and negative samples were run to validate the assay.

3.3   The validation results on the LC480

In total, 73 patients with 136 lymph nodes were run on the LC480 assay. The following sections describe the results of the LC480 by comparing the Cq values to those of the GeneSearch assay.

3.3.1   The positive nodes

Thirty-six lymph nodes from twenty patients were found to be positive by both assays. There were no discordant results between GeneSearch and the LC480 assay. The same cut-offs of the markers PBGD, CK19 and MGB that were used in GeneSearch were also applied to the LC480 assay. The table of all samples with their Cq values can be found in the appendix (pg 200).

The following three scatter graphs illustrate the difference in Cq values for all three markers for each positive node.
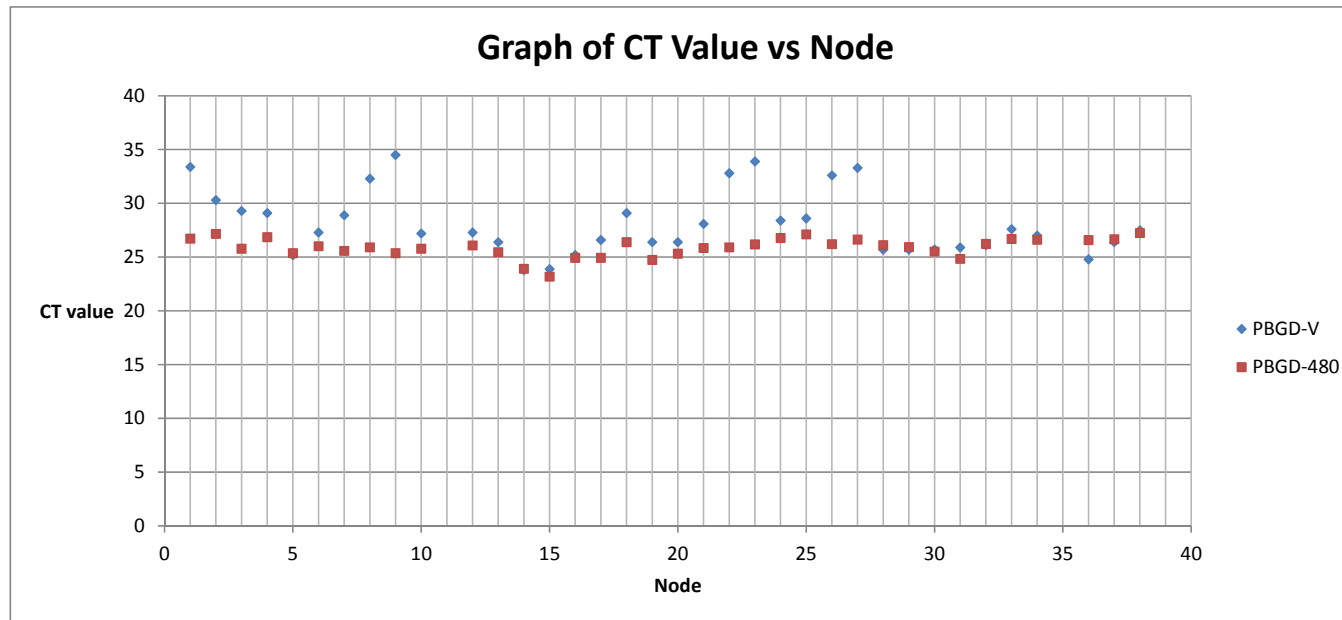
Graph 1.1 Comparing GeneSearch PBGD Cq value with LC480 PBGD Cq value for positive nodes (V = GeneSearch, 480 = LC480)

In order for the results of a node to be valid, PBGD, the reference gene, should have a Cq value of less than or equal to 36 according to the GeneSearch. All the nodes tested by the LC480 achieved PBGD Cq values of less than or equal to 36.
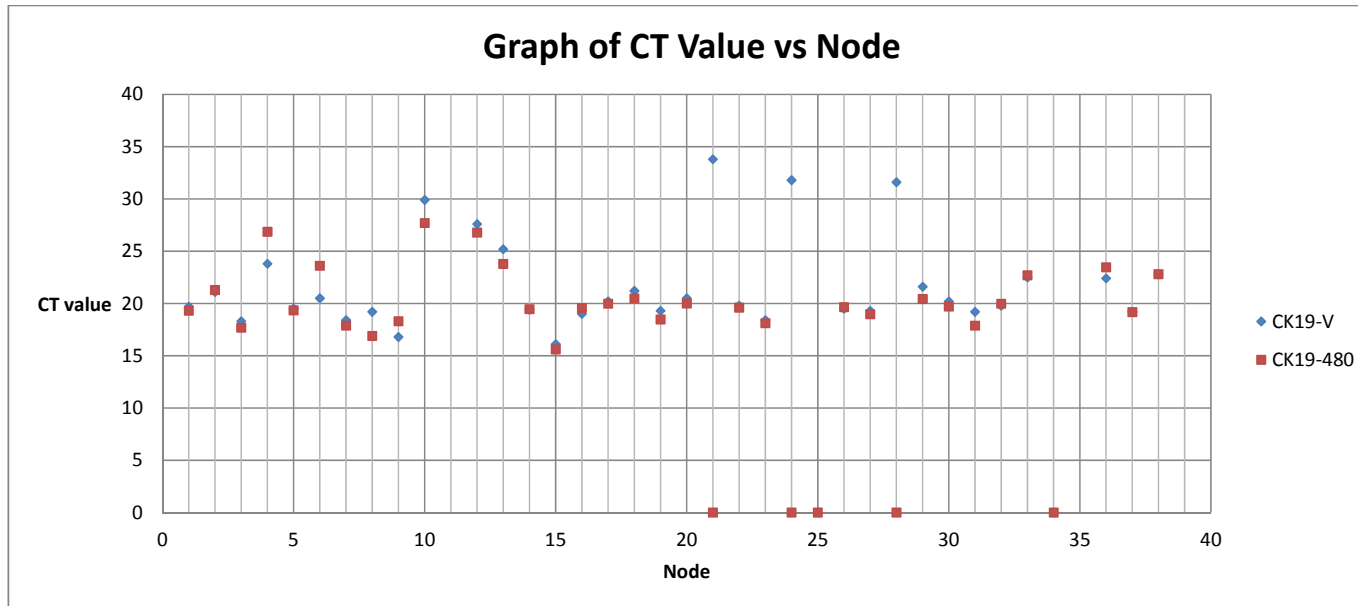
Graph 1.2 Comparing GeneSearch CK19 Cq value with LC480 CK19 Cq value for positive nodes (V = GeneSearch, 480 = LC480

The majority of nodes run on the LC480 had Cq values for CK19 similar to those of the GeneSearch. However, three lymph nodes that were positive for CK19 using GeneSearch BLN assay were negative on the LC480. No nodes were found to be positive on the LC480 but negative by GeneSearch
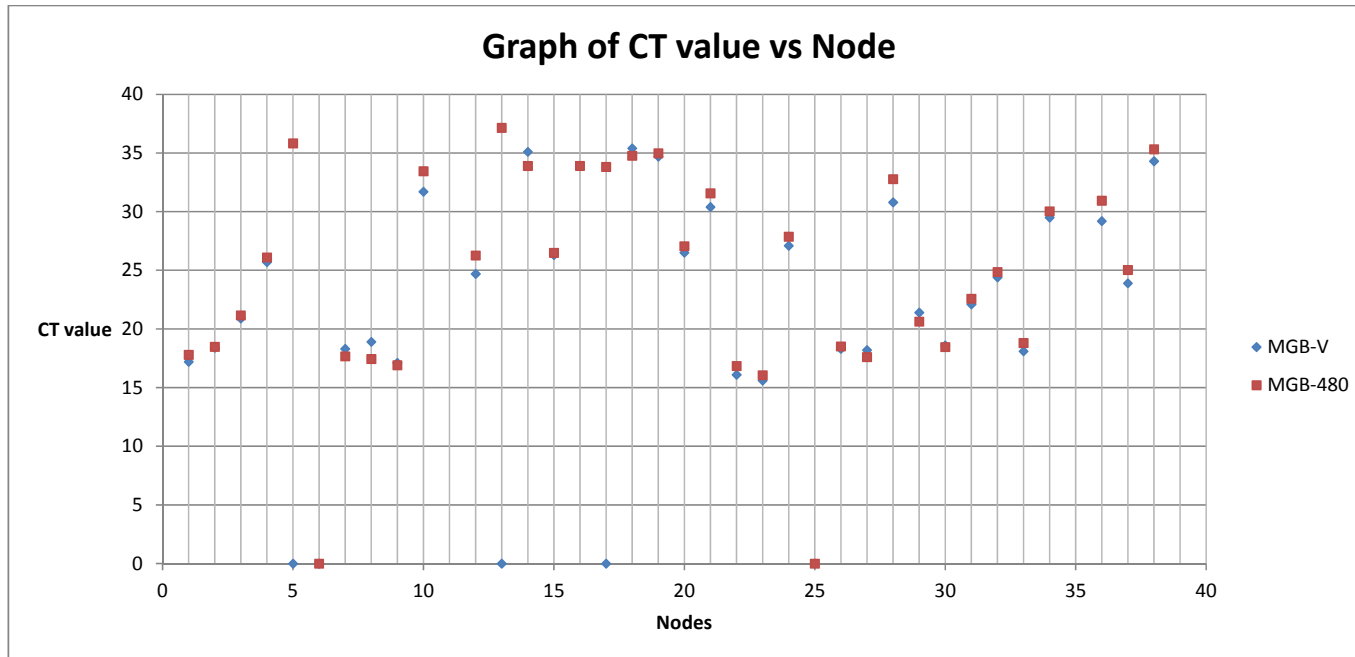
Graph 1.3. Comparing GeneSearch MGB Cq value with LC480 MGB Cq value for positive nodes (V = GeneSearch, 480 = LC480)

All nodes were found to have the same results for MGB. Several nodes were found to have Cq values of 0 with GeneSearch but registered a Cq value, albeit higher than the cut-off of 31, with the LC480.

In summary, there were no discrepancies in the results of the positive nodes overall, however, three nodes found to be positive for CK19 on GeneSearch were negative on the LC480. Ck19 detection on the LC480 is less sensitive than on the GeneSearch assay.

3.3.2   The negative nodes

Ninety-nine negative nodes from fifty-two patients were tested and no discordant results between GeneSearch and the LC480 were found. For the table showing the Cq values of all the negative cases please see the appendix (pg 202).

3.3.3   Discordant case

There were no discordant cases between GeneSearch and the LC480. However, there was one discordant case (12395B) when comparing histology with both assays. A 3mm macrometastasis was found in one of two slices taken for histology. The GeneSearch and LC480 Cq values for CK-19 and MGB were 0 for both. A full explanation is available in the discussion chapter.

3.4   Reducing the run time

The original run time using the recommended run protocol was found to be 52 minutes. This was far too long for an intra-operative assay and so attempts to reduce the run time were done.

The table below shows the original run parameters on the LC480

	Temperature (°C)	Hold (seconds)	Number of Cycles
Reverse Transcription	63	180	1
Denaturation	95	30	1
Amplification	95	10	45
	60	30	
	72	1	
Cooling	40	10	1

Table 18. The original run parameters on the LC480

The sample used was previously found to be positive on the LC480 for MGB, with a Cq value of 29, and negative for CK19, with a Cq value of 0. The Cq value of PBGD was 25. The Cq values of the experimental runs are shown along with the time taken for the run.

First run

Amplification 60 °C for _30 s_ for 35 cycles. Run time: **52 minutes**

PBGD	CK19	MGB
26.62	ND	30.03

Table 19.1. The Cq values of each marker (60°C for 30s), ND = not detected

Second run

Amplification 60 °C for _20 s_ for 35 cycles. Run time: **48 minutes 28 seconds**

PBGD	CK19	MGB
26.86	ND	32.68
26.75	ND	33.20

Table 19.2. The Cq values of each marker (60°C for 20s), ND = not detected

Third run

Amplification 60 °C for *10 s* for 35 cycles. Run time: **43 minutes 28 seconds**

PBGD	CK19	MGB
ND	ND	ND
ND	ND	ND

 ND = not detected

Table 19.3. The Cq values of each marker (60°C for 10s)

In the third run, no markers were detected. The fastest possible time was still too long at 48 minutes and 28 seconds.

Fourth run: Turbo PCR

1. Amplification 60 °C for 30 s: 10 cycles (1 cycle takes 1:38)
2. Amplification 60 °C for 20 s: 10 cycles (1 cycle takes 1:12)
3. Amplification 60 °C for 10 s: 15 cycles (1 cycle takes 1:03)

PBGD	CK19	MGB
ND	ND	ND
ND	ND	ND
ND	ND	ND

ND = not detected

Table 19.4. The Cq values of each marker (Turbo PCR)

No Cq values were obtained.

Despite attempts at reducing the run time, the assay still took far too long and failed to detect the markers. The decision was then made to change the platform from the LC480 and instead use the SmartCycler machine. GeneSearch also uses this platform and so an assay time comparable to GeneSearch was already known to be achievable.

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Chapter 4   Results on the SmartCycler platform - Optimisation

The Metasin assay was optimised on the SmartCycler platform and the run time reduced to a time comparable to that of GeneSearch by reducing the denaturing time and running turbo PCR. The assay was optimised by changing the primer concentration from 1.5µM to 2.5µM, increasing the RNA volume from 1µl to 5µl and increasing the RT step from 120s to 300s. Once the assay was optimised, all the samples had their RNA extracted using the RNeasy Mini kit (Qiagen) and the RNA run using the new protocol.

4.1   Results of the pilot runs

The first pilot run used multiplexed markers using known positive samples. The Cq values are shown in the following tables and compared to the Cq values of GeneSearch for the same samples.

Sample	Marker	Metasin Cq value	GeneSearch Cq value
745	PBGD	25.2	26.4
	CK19	17.9	19.2
	MGB	23.8	23.9
13447	PBGD	24.8	32.6
	CK19	17.8	19.5
	MGB	17.6	18.3

Table 20.1 Cq values of two known positive samples compared with GeneSearch from the first pilot run.

The results show that the samples had similar Cq values. Further nodes, including negative nodes, were tested in a second run and the Cq values compared.

The table below compares the Cq values of Metasin (M) and GeneSearch (G) of both positive and negative samples.

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	PBGD M	PBGD G	CK19 M	CK19 G	MGB M	MGB G
8759F	23.4	26.6	20.1	20.2	34.3	0
8759F	23.5	26.6	19.9	20.2	33.8	0
10762E	25.7	33.9	18.1	18.4	16.1	16.1
10762E	25.6	33.9	18.7	18.4	16.8	16.1
18472C	26.1	27.8	0	0	0	0
18472C	26.5	27.8	0	0	0	0
1978B	27.7	28	0	0	0	0
1978C	26.2	26.6	0	0	0	0
1978D	25.9	26.5	0	0	0	0

Table 20.2. Cq values of known positive and negative samples compared with GeneSearch from the second pilot run (M = Metasin, G = GeneSearch).

The second run showed the Metasin results to be more sensitive than the GeneSearch results. No discordant results between the assays were recorded.

4.2   Results of optimising the Metasin assay and reducing the run time

The run time was reduced by changing the denaturing and annealing time as well as the RT step from 300s to 120s. The time was reduced to a time comparable to that of GeneSearch ie approximately 25 minutes for one node. The assay was also optimised again by changing the primer concentration from 1.5 to 2 to 2.5µM and RNA volume from 1µl to 2.5, 3, 4 and 5µl. It was found that the optimum primer concentration and RNA volume was 2.5µM and 4µl respectively.

Commented [SP2]: You have nott amended as requested to include details of changes; ie range of concetrations and RNA vol tried.

4.2.1   Reducing the run time

The GeneSearch result of the positive node 16039C is given so as to compare the Cq values with those from the runs when changing the run protocol.

16039C		
PBGD	**CK19**	**MGB**
25.7	20.2	18.6

Table 21. The Cq values of each marker for the positive sample 16039C with Genesearch

2.5µl of RNA were used in these runs. The previously optimised primer and probe concentrations of 1.0µM and 0.1µM were used.

Changing the denaturation time

The denaturation temperature is set at 95°C. The time taken for the denaturation step of the PCR reaction was changed ranging from 1s to 10s and the Cq values compared to those of GeneSearch.

Temperature (°C)	Seconds	Temperature (°C)	Seconds	PBGD	CK19	MGB
60	30	95	1	26.5	19.8	23.4
60	30	95	2	26.5	21.7	22.2
60	30	95	4	26.7	21.8	22.3
60	30	95	6	26.6	21.7	22.2
60	30	95	8	26.1	21.8	22.3
60	30	95	10	26.3	21.9	22.3

Table 22.1 The Cq values of sample 16039C when changing the denaturing time

No significant change in Cq values was observed when changing the time of denaturation. Therefore, the 95°C step was reduced to 1s.

Changing the annealing time

The annealing temperature is set at 60°C. The time taken for the annealing step of the PCR reaction was changed ranging from 6s to 30s and the Cq values compared to those of GeneSearch.

Temperature (°C)	Seconds	Temperature (°C)	Seconds	PBGD	CK19	MGB
60	6	95	10	0	34.5	0
60	10	95	10	31.2	28.6	29.5
60	15	95	10	28.5	25.4	25.8
60	20	95	10	27.4	23.4	24.4
60	25	95	10	26.8	22.4	22.6
60	30	95	10	26.2	21.7	22.4

Table 22.2 The Cq values of sample 16039C when changing the annealing time

Reducing the 60°C step drastically changed the Cq values. It was decided to try and optimise the assay further in order to reduce the annealing time.

4.2.2 Changing the RNA volume

The RNA volume of the sample 16039C was changed using 1μl, 2.5μl, 3μl, 4μl and 5μl volumes. The optimised run parameter of 1s at 95°C and the original run time of 30s at 60°C for 35 cycles were used to try and determine the optimum RNA volume.

The results of the Cq values for each marker are given.

RNA volume (μl)	PBGD	CK19	MGB
1	27.6	22.2	21.8
2.5	28.3	22.5	22.5
3	27.7	22.4	21.5
4	27.5	21.7	20.3
5	28.9	23.4	22

Table 23 Changing the RNA volume

The run shows that by increasing the RNA volume to 4µl, the Cq values are closest to those of GeneSearch for those run parameters. However, the assay took 28 minutes which is still longer than the GeneSearch time of 25 minutes.

4.2.3 Turbo PCR results

In an attempt to reduce the run time from 28 minutes, the annealing time was reduced in a step-wise fashion.

The following two positive samples were used in these runs.

GeneSearch results of the samples to compare with those of the experimental runs

Sample	PBGD	CK19	MGB
17502B	25.9	19.2	22.1
18243B	27.6	22.5	18.1

Table 24 The GeneSearch Cq values of two positive samples

The results of the three turbo runs, along with their run times, are given in the following tables.

Turbo run 1

Sample	PBGD	CK19	MGB
17502B	28.2	21.3	28.4
18243B	29.3	26.4	22.1

Table 25.1 Cq values of turbo run 1

Run time of 17:10:01 – 17:35:14 (25 mins 13s)

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Turbo run 2

Sample	PBGD	CK19	MGB
17502B	26.8	19.7	24.4
18243B	28.4	24.8	19.1

Table 25.2 Cq values of turbo run 2

Run time of 17:13:24 – 17:38:52 (25 mins 28s)

Turbo run 3

Sample	PBGD	CK19	MGB
17502B	31.5	22.9	30.7
18243B	32.8	30.1	24.5

Table 25.3 Cq values of turbo run 3

Run time of 17:15:05 – 17:38:55 (23 mins 50s)

The run protocol of turbo 2 was the fastest and did not compromise the Cq values.

4.2.4   Changing the RT step

The RT step was increased in order to improve the Cq values. Two known positive samples were used.

The table shows the Cq values of the samples used when changing the RT step

Sample	PBGD	CK19	MGB
16039C	25.7	20.2	18.6
13447E	33.3	19.3	18.2

Table 26 The Cq values of the two positive samples from GeneSearch

The following table shows the Cq values for each marker for the samples and their associated RT time.

RT time (s)	Sample	PBGD	CK19	MGB
120	16039C	25.4	20	18.5
120	13447E	26.2	19.7	17.6
240	16039C	24.6	17.1	17.2
240	13447E	25.5	16.1	17
300	16039C	24.3	15.7	17.6
300	13447E	24.7	15.7	17.2

Table 27 Increasing the RT step

The results show no significant difference in the Cq values and so a time of 240s was chosen.

4.2.5 Changing primer concentration

The primer concentration was changed in order to improve the Cq values of the samples, making them comparable to those obtained by GeneSearch.

Sample = 16039C			
Primer concentration (µM)	**PBGD**	**CK19**	**MGB**
1.5	24.7	17.2	18.1
2	22.2	17.5	17.7
2.5	23.7	17.1	17.5

Table 28.1 Comparing the Cq values for primer concentrations 1.5, 2 and 2.5µM for sample 16039C

Sample = 17502B			
Primer concentration (µM)	**PBGD**	**CK19**	**MGB**
1.5	25.3	16.7	21.9
2	25.1	16.6	21.6
2.5	25.3	16	21.2

Table 28.2 Comparing the Cq values for primer concentrations 1.5, 2 and 2.5µM for sample 17502B

2.5µM was chosen as the optimum primer concentration.

4.2.6   Qiagen extracted RNA vs GeneSearch extracted RNA

Up to this point, the RNA used in the optimisation runs had been extracted using GeneSearch materials. Once the assay optimisation and run time had been reduced, the extraction of RNA was carried out using the RNEasy Mini kit (Qiagen) and the results compared.

The table compares the Cq value of each marker with RNA extracted using GeneSearch and Qiagen extraction kits. Two negative samples and two positive samples were compared.

Sample	PBGD Q	PBGD G	CK19 Q	CK19 G	MGB Q	MGB G
2618A	29.6	29.4	0	0	0	0
2618B	30	30.4	0	0	0	0
3256E	25.1	26.5	28.9	26.8	31.2	30.5
3258A2	24	24.8	18.9	20.4	31.1	30.3

Table 29 Comparing the Cq values of RNA extracted using either GeneSearch or Qiagen extraction kits, Q = Qiagen, G = GeneSearch.

The Cq values are very similar indicating there is no significant difference between Qiagen extracted RNA and GeneSearch extracted RNA.

In concluding this chapter, the optimum primer concentration and RNA volume is 2.5µM and 4µl respectively. The selection of certain concentrations and volumes was influenced mainly by the ease at which calculations could be made and the volumes could be pipette as well as by financial constraints of trying to limit the amount of reagents used.

The optimum run protocol of the Metasin assay, with an average run time of 25 minutes, is shown in the following table.

	Temperature (°C)	Seconds	Number of Cycles
Reverse Transcription	63	240	1
Denaturation	95	30	1
Amplification 1	95	1	
	60	30	
	72	1	10
Amplification 2	95	1	
	60	10	
	72	1	25
Cooling	40	10	1

Table 30 The optimum run protocol of Metasin

Chapter 5   The Metasin validation results

Once the optimisation of the assay and the run time were improved, all the samples had their RNA extracted using Qiagen's RNeasy Mini kit RNA extraction kit and run on the Metasin in-house assay. This chapter sets out the results obtained from the Metasin assay by first analysing the positive results, then the negative results and finally the earlier results from the LC480 were also compared to those of Metasin.

5.1 Positive patients and nodes

Out of 154 cases (352 nodes) there were 37 positive cases (64 positive concordant nodes) for both GeneSearch and Metasin, 24% of the total number of cases and 18% of the total number of nodes. A full list of the Cq values of these nodes are available in the appendix (pg 208).
The following three scatter graphs compare the Cq values of the positive nodes of GeneSearch and Metasin. The Cq values of both assays were plotted against the node identification number on the same vertical line.
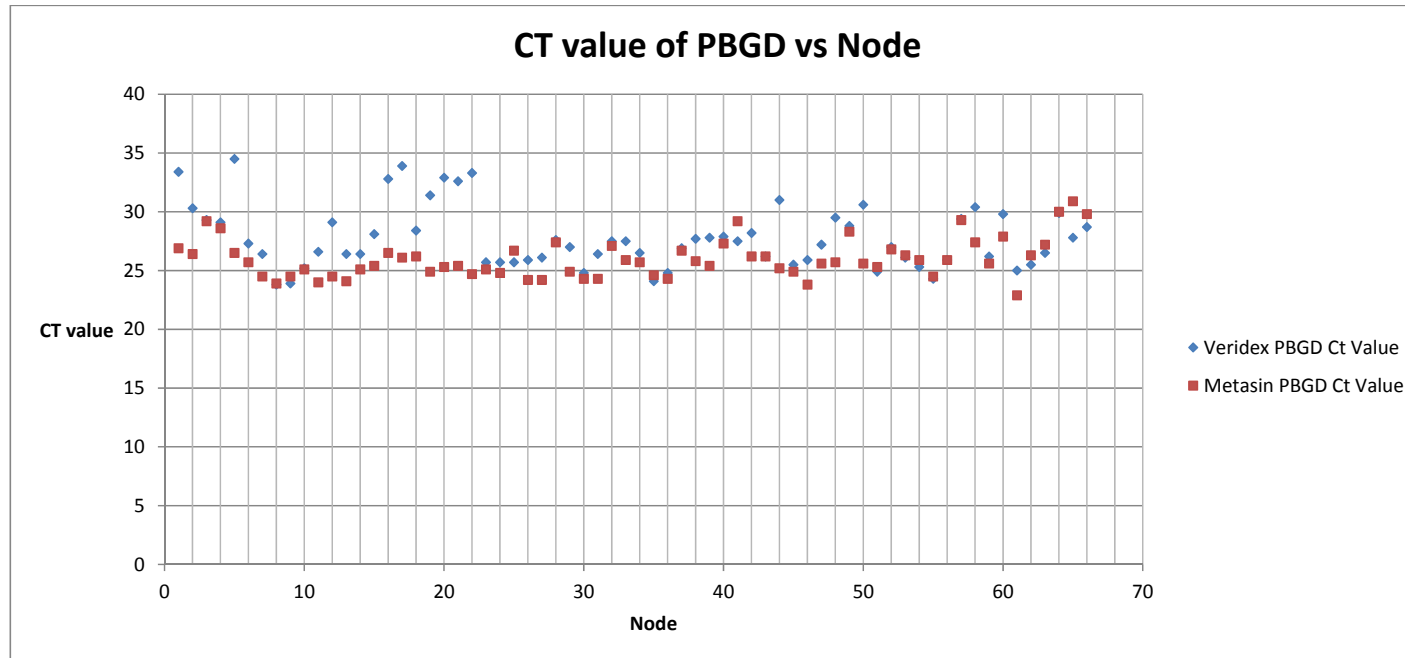
Graph 2.1 Comparing GeneSearch and Metasin Cq values of PBGD of the positive nodes (Veridex = GeneSearch)
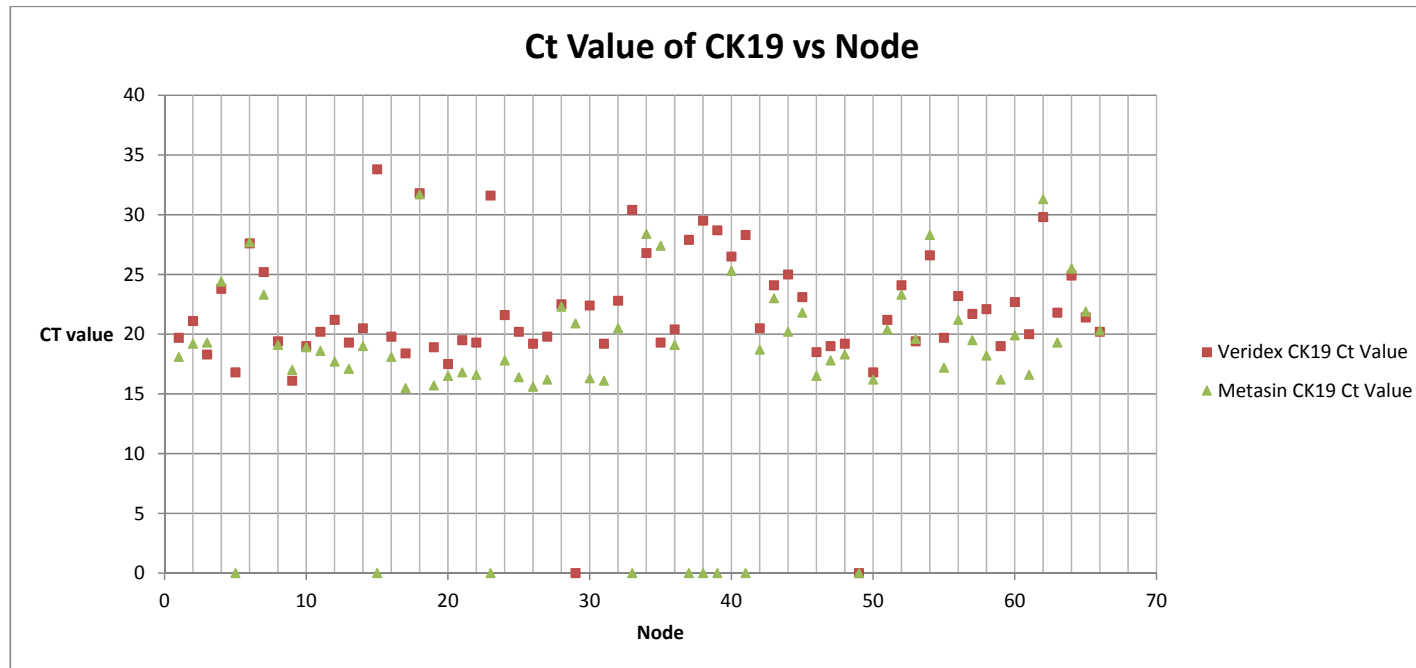
Graph 2.2 Comparing GeneSearch and Metasin Cq values of CK19 of the positive nodes (Veridex = GeneSearch)
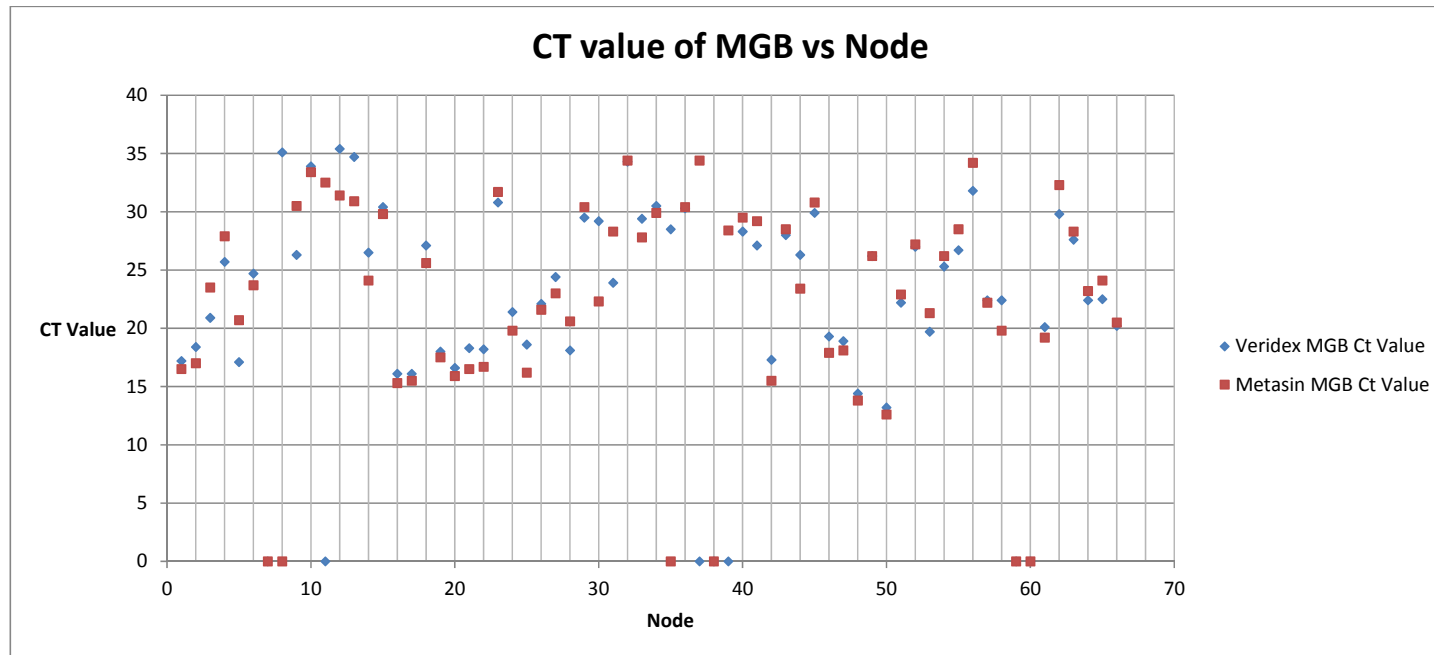
Graph 2.3 Comparing GeneSearch and Metasin Cq values of MGB of the positive node (Veridex = GeneSearch)

5.2 Negative patients and nodes

For the table showing the Cq values of the negative cases please see the appendix (pg 210).

In total, 111 patients with 280 nodes were found to be negative for metastatic breast cancer by both Metasin and GeneSearch.

The following scattergraph plots the GeneSearch results against the Metasin results of the negative cases for PBGD.
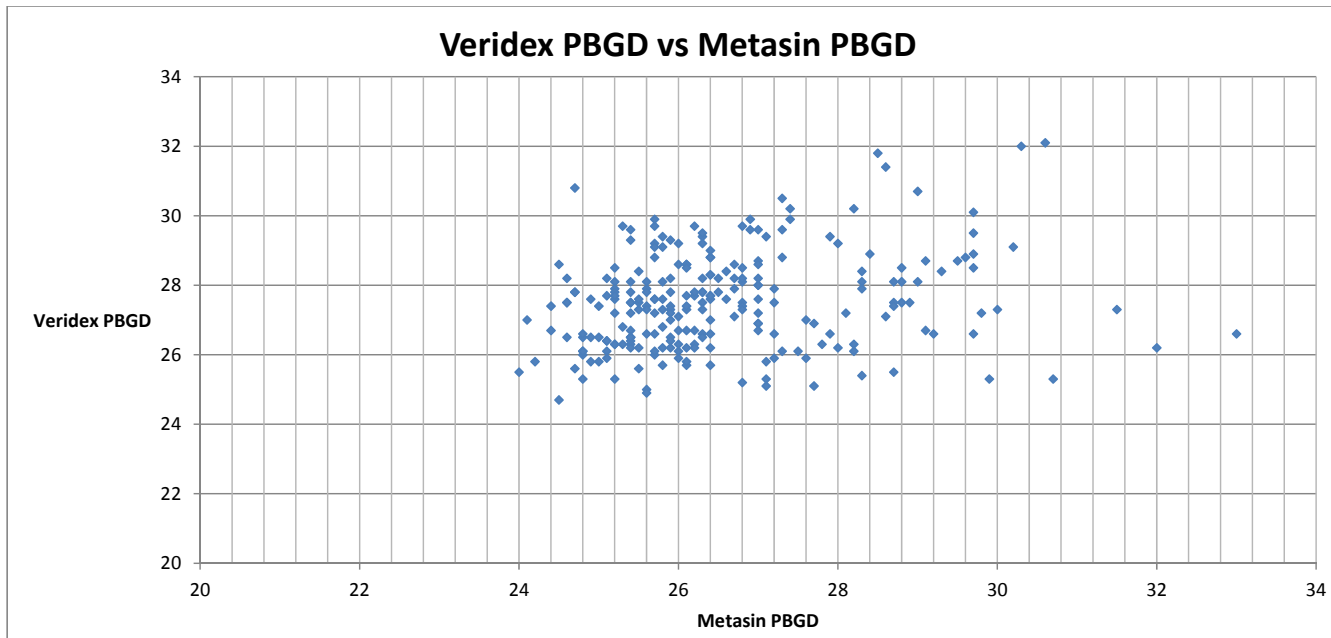
Graph 3 Comparing GeneSearch and Metasin Cq values of PBGD of the negative nodes (Veridex = GeneSearch)

The PBGD graph shows a concentrated small area showing that the Cq values of the reference gene for both assays are similar.

5.3 Comparing the Metasin and LC480 results

The Metasin Cq values of all three markers in 18 positive cases with 32 nodes were compared to those of the LC480.

There were five nodes where CK19 disagrees, 3 nodes where the CK19 with the LC480 is positive and 2 nodes where Metasin is positive.

There were 3 nodes where MGB disagreed. All three were positive for Metasin, however, they were picked up at a slightly higher CT value with LC480.

The full table showing the Cq values is available in the appendix (pg 224).

The following scatter graphs compare the Cq values of the three markers.
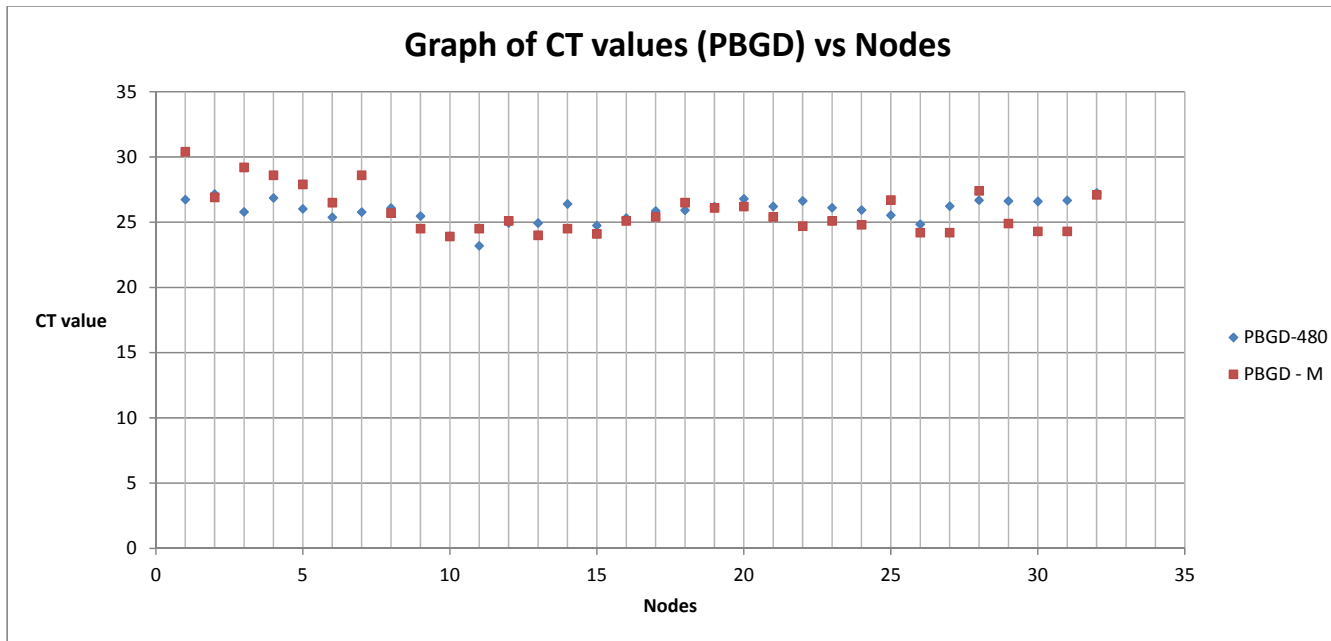
Graph 4.1 Comparing the Cq values of positive nodes of the LC480 and Metasin assay for PBGD
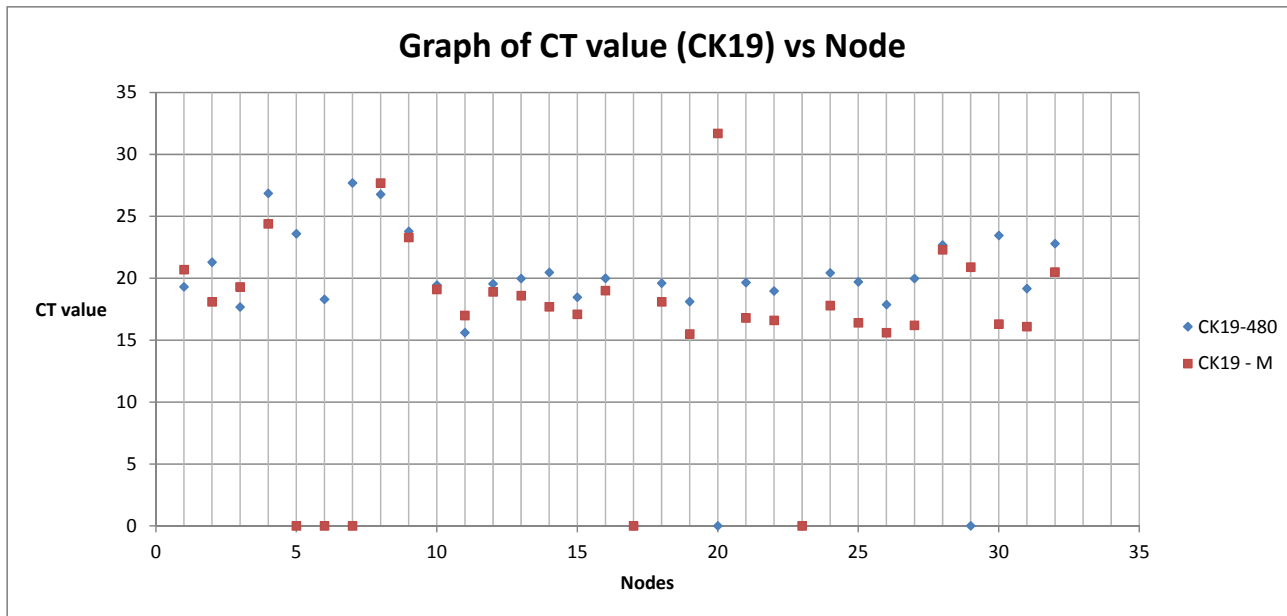
Graph 4.2 Comparing the Cq values of positive nodes of the LC480 and Metasin assay for CK19
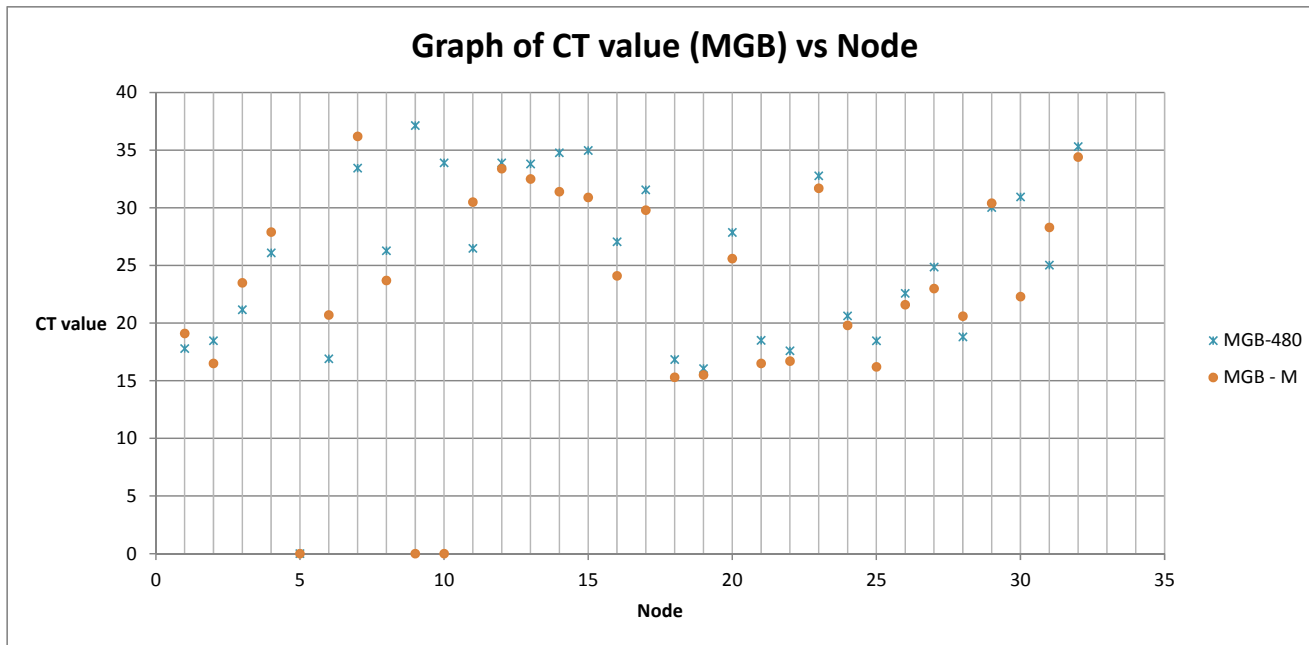
Graph 4.3 Comparing the Cq values of positive nodes of the LC480 and Metasin assay for MGB

The scattergraphs show the majority of nodes have similar Cq values.

Chapter 6   Statistical analysis results

Statistical tests were employed to determine the cut-offs for positive and negative nodes as well as to distinguish macrometastases from micrometastases.

The main objectives to be determined statistically were:

1. Cut-off Cq values for CK19 and MGB for positive and negative nodes
2. Cut-off Cq values for CK19 and MGB for macrometastases and micrometastases
3. The sensitivity and specificity of the assay
4. The positive and negative predictive value of the assay

6.1 Proposed Cut-off values for positive and negative nodes: comparing sensitivities and specificities

The results of the proposed cut-off values, along with the sensitivities and specificities for both CK19 and MGB, are tabulated.

The following six tables show the results of the number of <u>nodes</u> with the proposed Cq cut-off value for CK19.

	Metasin	
GeneSearch	Positive	Negative
Positive	53	9
Negative	1	289

Table 31.1: nodes results for a CK19 cut-off value of 28

	Metasin	
GeneSearch	Positive	Negative
Positive	55	7
Negative	1	289

Table 31.2: nodes results for a CK19 cut-off value of 29

	Metasin	
GeneSearch	Positive	Negative
Positive	55	7
Negative	3	287

Table 31.3: nodes results for a CK19 cut-off value of 30

	Metasin	
GeneSearch	Positive	Negative
Positive	55	7
Negative	3	287

Table 31.4: nodes results for a CK19 cut-off value of 31

	Metasin	
GeneSearch	Positive	Negative
Positive	56	6
Negative	4	286

Table 31.5: nodes results for a CK19 cut-off value of 32

	Metasin	
GeneSearch	Positive	Negative
Positive	56	6
Negative	4	286

Table 31.6: nodes results for a CK19 cut-off value of 33

Proposed CK19 cut off value	Sensitivity (95% CI)	Specificity
28	85% (76%-94%)	103%
29	89% (81%-97%)	102%
30	89% (81%-97%)	101%
31	89% (81%-97%)	101%
32	90% (82%-97%)	100%
33	90% (82%-97%)	100%

Table 32: the sensitivities and specificities for each proposed Cq cut-off value for CK19
nodes

By adopting a cut-off of 28 for CK19, there would be too many false negatives and the
sensitivity is the lowest at 85%. At a cut-off of 30 or 31, two cases become Metasin
positive but GeneSearch negative. When assessing the histology results for these two
cases, they are found to be positive and therefore represent true positives which were
missed by GeneSearch. For cut offs 32 and 33, an extra case is added to the Metasin

positive and GeneSearch negative box which to make a total of 4 nodes. The fourth node was also shown to be positive on histology. The sensitivity of 90% is highest for cut-offs 32 and 33. For these cut-offs, there are also less false negatives. The cut offs of 32 and 33 have the best sensitivities and specificities.

The following six tables show the results of the <u>cases</u> with the proposed Cq cut-off value for CK19.

	Metasin	
GeneSearch	Positive	Negative
Positive	30	5
Negative	0	119

Table 33.1: cases results for a CK19 cut-off value of 28

	Metasin	
GeneSearch	Positive	Negative
Positive	31	4
Negative	0	119

Table 33.2: cases results for a CK19 cut-off value of 29

	Metasin	
GeneSearch	Positive	Negative
Positive	31	4
Negative	2	117

Table 33.3: cases results for a CK19 cut-off value of 30

	Metasin	
GeneSearch	Positive	Negative
Positive	31	4
Negative	2	117

Table 33.4: cases results for a CK19 cut-off value of 31

	Metasin	
GeneSearch	Positive	Negative
Positive	32	3
Negative	3	116

Table 33.5: cases results for a CK19 cut-off value of 32

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	Metasin	
GeneSearch	Positive	Negative
Positive	32	3
Negative	3	116

Table 33.6: cases results for a CK19 cut-off value of 33

Proposed cut off	Sensitivity (95% CI)	Specificity
28	86% (74%-97%)	104%
29	88% (77%-99%)	103%
30	88% (77%-99%)	101%
31	88% (77%-99%)	101%
32	91% (82%-100%)	100%
33	91% (82%-100%)	100%

Table 34: the sensitivities and specificities for each proposed Cq cut-off value for CK19 cases

By looking at the cases with cut-offs of 32 and 33, these had the best sensitivity of 91% and specificitiy of 100%. Therefore, the best cut-off for CK19 can be chosen as either 32 or 33.

The same analysis was done for MGB.

The following six tables show the results of the nodes with the proposed Cq cut-off value for MGB.

	Metasin	
GeneSearch	Positive	Negative
Positive	42	11
Negative	1	298

Table 35.1: nodes results for a MGB cut-off value of 29

	Metasin	
GeneSearch	Positive	Negative
Positive	46	7
Negative	2	297

Table 35.2: nodes results for a MGB cut-off value of 30

	Metasin	
GeneSearch	Positive	Negative
Positive	50	3
Negative	4	295

Table 35.3: nodes results for a MGB cut-off value of 31

	Metasin	
GeneSearch	Positive	Negative
Positive	51	2
Negative	6	293

Table 35.4: nodes results for a MGB cut-off value of 32

	Metasin	
GeneSearch	Positive	Negative
Positive	52	1
Negative	10	289

Table 35.5: nodes results for a MGB cut-off value of 33

	Metasin	
GeneSearch	Positive	Negative
Positive	52	1
Negative	11	288

Table 35.6: nodes results for a MGB cut-off value of 34

Proposed cut off	Sensitivity (95% CI)	Specificity
29	79% (68%-90%)	103%
30	87% (78%-96%)	102%
31	94% (87%-100%)	99%
32	96% (91%-100%)	99%
33	98% (94%-100%)	97%
34	98% (94%-100%)	97%

Table 36: the sensitivities and specificities for each proposed Cq cut-off value for MGB nodes

By adopting cut-offs of 29 and 30, there are too many false negatives with sensitivities of 79% and 87% respectively. The higher the cut-off value, the higher the number of nodes that become Metasin positive and GeneSearch negative. With a cut-off of 34, 11 nodes are Metasin positive and GeneSearch negative. 5 of these nodes were shown to be negative on histology and therefore introduces 5 false positive nodes. The

specificity is also lower at 97%. It appears that having a cut-off of 32 or 33 would be the best values when balancing sensitivity and specificity.

Similar analysis was carried out using the number of cases rather than nodes.

	Metasin	
GeneSearch	Positive	Negative
Positive	30	5
Negative	0	119

Table 37.1: cases results for a MGB cut-off value of 29

	Metasin	
GeneSearch	Positive	Negative
Positive	33	2
Negative	1	118

Table 37.2: cases results for a MGB cut-off value of 30

	Metasin	
GeneSearch	Positive	Negative
Positive	33	2
Negative	2	117

Table 37.3: cases results for a MGB cut-off value of 31

	Metasin	
GeneSearch	Positive	Negative
Positive	34	1
Negative	3	116

Table 37.4: cases results for a MGB cut-off value of 32

	Metasin	
GeneSearch	Positive	Negative
Positive	35	0
Negative	5	114

Table 37.5: cases results for a MGB cut-off value of 33

	Metasin	
GeneSearch	Positive	Negative
Positive	35	0
Negative	5	114

Table 37.6: cases results for a MGB cut-off value of 34

Proposed cut off	Sensitivity (95% CI)	Specificity
29	86% (74%-97%)	104%
30	94% (86%-100%)	100%
31	94% (86%-100%)	100%
32	97% (91%-100%)	98%
33	100%	96%
34	100%	96%

Table 38: the sensitivities and specificities for each proposed Cq cut-off value for MGB cases

By adopting a cut-off of 32 for MGB, there would be less false positives introduced than cut-offs for 33 and 34. The specificity is also higher at 98% compared to 96% for the cut-offs 33 and 34.

In summary, the optimum cut-off values for CK19 lie between 32 and 33 and for MGB around 32.

6.2 Establishing the cut off points for positive and negative nodes and the cut- off points for macrometastasis and micrometastasis: Scatterplot

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Cut-off values
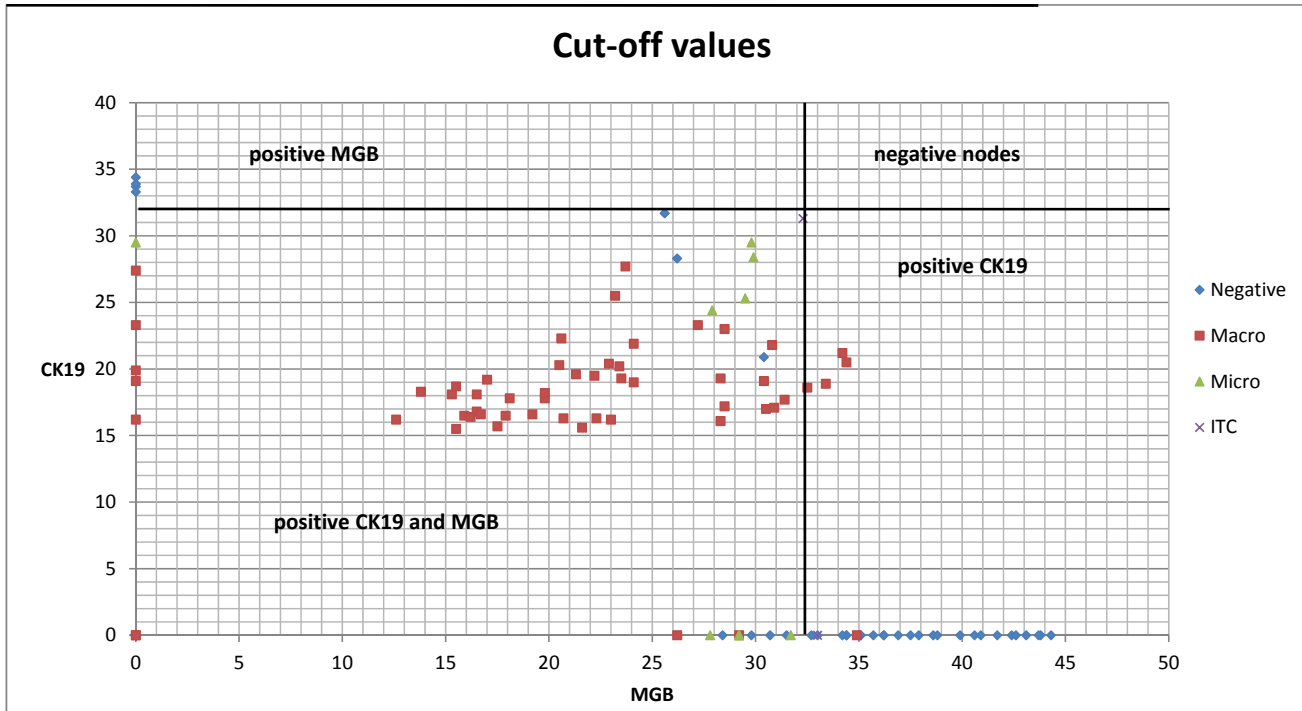
Graph 5. The graph plots CK19 vs MGB for all positive nodes and is colour coded according to histopathological size of the metastatic deposit.

Once all the GeneSearch positive and negative samples were run on Metasin, the Cq values were plotted on the graph above and the lines drawn to separate the positive and negative nodes. Extrapolation from the graph identified the optimum cut-off Cq values for positive and negative nodes on Metasin to be:

CK19 = 32

MGB = 32.3

The optimum cut-offs established in the previous section (6.1) were around 32 for MGB and 32-33 for CK19. The optimum cut-offs extrapolated from the scatterplot is consistent with these findings.

The number of patients used in the statistical analysis for determining the cut-offs during the validation of the GeneSearch assay was 274, 120 more patients than were included in the Metasin data analysis. Therefore, it was decided to adopt the cut-offs of 32 and 32.3 respectively for CK19 and MGB (taken from the scatterplot above) until the number of cases run on Metasin was at least at large, or larger, than 274 cases and when review with a larger dataset could be carried out.

By adopting the cut offs of 32 and 32.3 for CK19 and MGB respectively, the sensitivity and specificity as well as the positive and negative predictive values of the Metasin assay are calculated for nodes and cases.

	Metasin	
GeneSearch	Positive	Negative
Positive	64	4
Negative	4	280

Table 39: showing the distribution of nodes

Sensitivity of Metasin is 94% (88% - 99%, 95% CI)

Specificity of Metasin is 100%

Positive predictive value is 94%

Negative predictive value is 98%

	Metasin	
GeneSearch	Positive	Negative
Positive	37	2
Negative	4	111

Table 40: showing the distribution of <u>cases</u>

Sensitivity of Metasin is 95% (88% - 100%, 95% CI)

Specificity of Metasin is 98%

Positive predictive value is 90%

Negative predictive value is 98%

In determining the cut-off values for distinguishing macrometastases and micrometastases, the same method was used by extrapolating from the scatterplot. A cut-off of 25 for CK19 and 26 for MGB were found to be consistent with the data.

The GeneSearch cut-offs for nodes containing micro- or macrometastases is 25 for CK19 and 26 for MGB. As the number of micrometastasis included in the Metasin data is fewer than 20 cases, the cut-offs of GeneSearch are adopted until a larger dataset is tested.

The table below compares the number of macro and micrometastases detected by Metasin and Histology for all concordant positive nodes.

	Metasin		
Histology	Macro	Micro	Total
Macro	50 (81.9%)	2 (3.2%)	52 (85.2%)
Micro	1 (1.6%)	8 (13%)	9 (14.7%)
Total	51 (84%)	10 (16.4%)	61 (100%)

Table 41: Comparison of tumour volumes in concordant positive nodes between Metasin and histology (n=61)

Out of 61 nodes, 50 were found to have macrometastasis by both Metasin and histology and 8 cases with micrometastasis for both. There were 2 cases which were found to be micrometastases by Metasin but macrometastases by histology and 1 case

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which was found to be a macrometastasis by Metasin but a micrometastasis on histology. These 3 discordant results are most likely due to tissue allocation bias caused by allocating alternate slices to the PCR assays and to histology.

The table below sumarises the cut-off values for both Metasin and GeneSearch assays.

Assay	Marker	Positive/Negative Cut-off	Macro- Micrometastasis Cut-off
GeneSearch	CK19	30	25
GeneSearch	MGB	31	26
Metasin	CK19	32	25
Metasin	MGB	32.3	26

Table 42 A comparison of the Cut off values of GeneSearch and Metasin

6.3 Comparing the sensitivity and specificity of Metasin and GeneSearch for the dataset

The sensitivity and specificity of GeneSearch is calculated in order to compare the assay's performance with Metasin. Histology is used as the gold standard when calculating the sensitivity and specificity of Veridex and GeneSearch is used as the gold standard when calculating the sensitivity and specificity of Metasin.

	Histology	
GeneSearch	Positive	Negative
Positive	60	8
Negative	5	279

Table 43: Nodes results when comparing GeneSearch with histology

Sensitivity of GeneSearch is 92% (85% - 98%, 95% CI)

Specificity of GeneSearch is 99%

	Histology	
GeneSearch	Positive	Negative
Positive	36	3
Negative	4	111

Table 44: Cases results when comparing GeneSearch with histology

Sensitivity of GeneSearch is 90% (81% - 99%, 95% CI)

Specificity of GeneSearch is 100%

The following two tables compare the sensitivity and specificity of Veridex and Metasin for the dataset.

	Sensitivity (95% CI)	Specificity
GeneSearch	92% (85% - 98%)	99%
Metasin	94% (88% - 99%)	100%

Table 45: Comparing the sensitivity and specificity of Metasin and GeneSearch by nodes

	Sensitivity (95% CI)	Specificity
GeneSearch	90% (81% - 99%)	100%
Metasin	95% (88% - 100%)	98%

Table 46: Comparing the sensitivity and specificity of Metasin and GeneSearch by cases

Metasin is shown to have a higher sensitivity than GeneSearch. However, these results use histology as the gold standard for GeneSearch and GeneSearch as the gold standard for Metasin. The sensitivity and specificity for Metasin when using histology as the gold standard is calculated and compared to GeneSearch.

	Histology	
Metasin	Positive	Negative
Positive	61	7
Negative	3	281

Table 47: Comparing Metasin with histology nodes results

Sensitivity of Metasin is 95% (89% - 100%, 95% CI)

Specificity of Metasin is 98%

	Histology	
Metasin	Positive	Negative
Positive	37	4
Negative	2	111

Table 48: Comparing Metasin with histology cases results

Sensitivity of Metasin is 95% (88% - 100%, 95% CI)

Specificity of Metasin is 98%

The following two tables compare sensitivity and specificity of Metasin and GeneSearch using histology as the gold standard for both

	Sensitivity (95% CI)	Specificity
GeneSearch	92% (85% - 98%)	99%
Metasin	95% (88% - 100%)	98%

Table 49: Comparing sensitivity and specificity of Metasin and GeneSearch using histology as the gold standard for both, nodes results

	Sensitivity (95% CI)	Specificity
GeneSearch	90% (81% - 99%)	100%
Metasin	95% (88% - 100%)	98%

Table 50: Comparing sensitivity and specificity of Metasin and GeneSearch using histology as the gold standard for both, cases results

In conclusion, Metasin is shown to have a higher sensitivity than GeneSearch for both nodes and cases.

6.4   Line of agreement

In order to appreciate the agreement between the two assays, the results of all nodes were plotted onto scatter graphs.
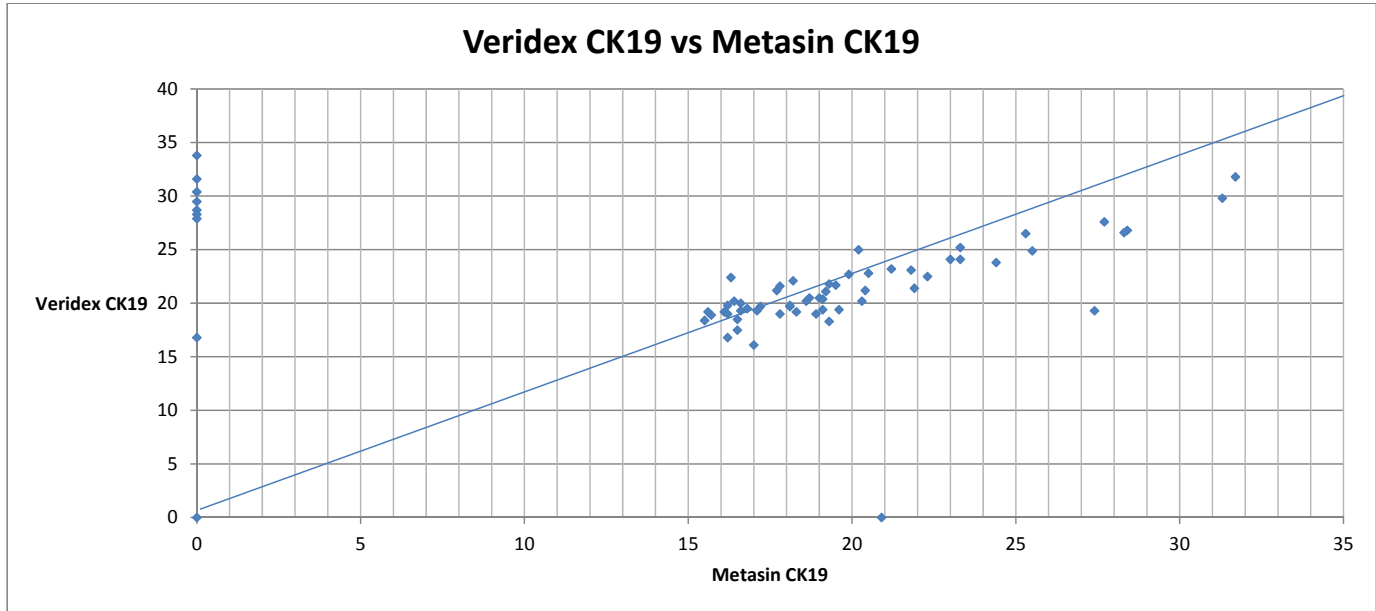
Graph 6.1 Plotting the Cq values of GeneSearch CK19 and Metasin CK19 (Veridex = GeneSearch)
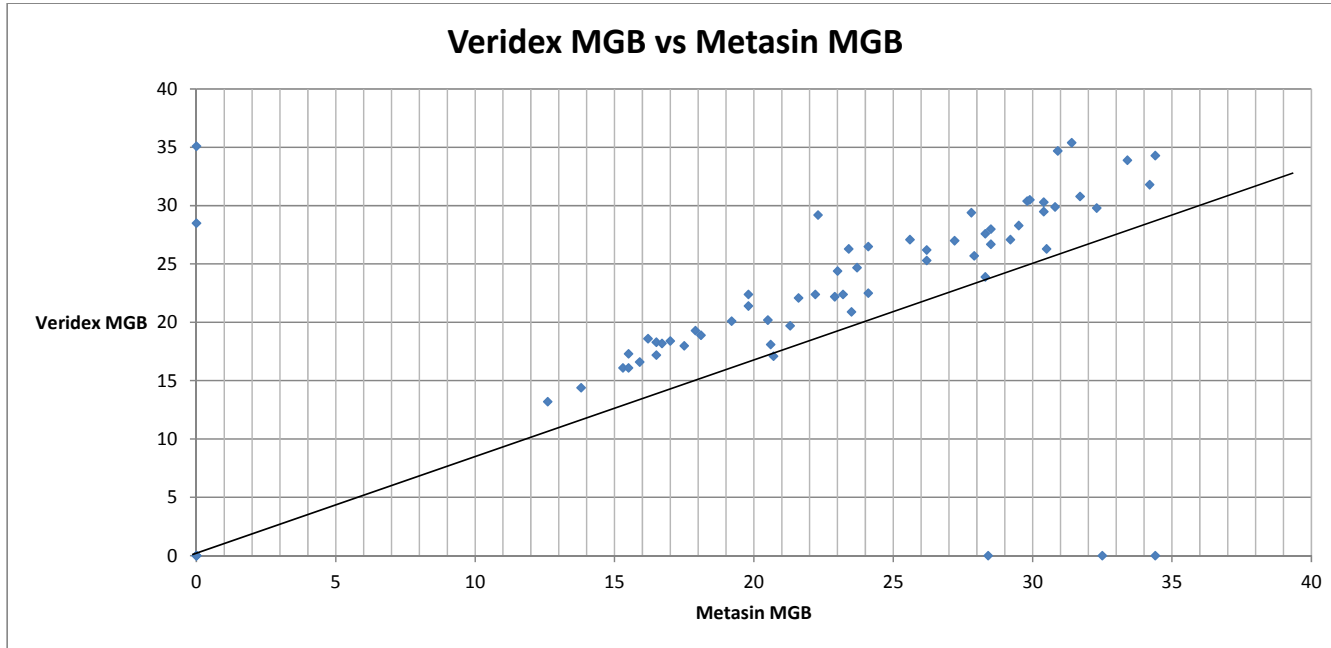
Graph 6.2 Plotting the Cq values of GeneSearch MGB and Metasin MGB (Veridex = GeneSearch)

When comparing the Cq values of both assays the following scattergraphs do not show a huge variation with the data points lying approximately along the 45° line.

6.5   Cohen's kappa

Out of 154 patients, 111 patients were found to be negative, 37 patients were positive and 6 patients were discordant for GeneSearch and Metasin. The value of Kappa is 0.90 and its confidence interval is 0.82 – 0.98, which does not include zero, showing the two assays significantly agree.

Observer A	GeneSearch
Observer B	Metasin

Observer B	Observer A		
	0	1	
0	111	2	113 (73.4%)
1	4	37	41 (26.6%)
	115 (74.7%)	39 (25.3%)	154

Weighted Kappa[a]	0.899
Standard error	0.040
95% CI	0.819 to 0.978

[a] Linear weights

Table 51. Cohen's kappa
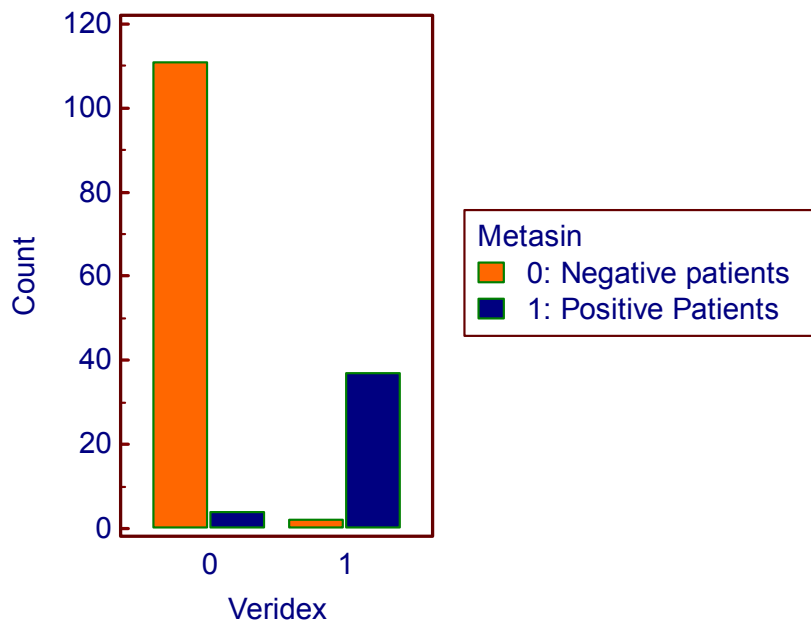
Figure 14. Distribution of concordant and discordant patients in Cohen's kappa

Chapter 7 - The discordant results

Out of 154 cases (352 nodes), there were 6 discordant cases (3.89%) when comparing Metasin and GeneSearch. There were 6 discordant nodes and 7 concordant nodes in the 6 cases. One positive concordant case with 5 nodes in total was found to have 3 positive concordant nodes and two discordant nodes. Therefore, out of 352 nodes, there were 8 discordant nodes in total. The aim of this chapter is to analyse more closely the discordant cases between Metasin and GeneSearch and compare with the histology results. The discordant cases were also tested using agarose gel electrophoresis and the results found to be inconclusive.

7.1 Comparing the results of GeneSearch, Metasin and Histology for the discordant cases

A summary of the number of nodes and cases of Metasin found to be concordant and discordant with histology is tabulated. The discordance rate is also shown. The analysis is divided into nodes and cases. The initial analysis includes nodes or cases that were originally negative on histology but which after further levels were done, were found to be positive.

Case based							
Concordant cases			**Discordant cases**		**Total**	**Discordant cases**	**Discordant cases %**
H+ M+		H- M-	H+ M-	H- M+			
Initial analysis	33	111	2	8	154	10	6.49%
Deeper levels	37	111	2	4	154	6	3.89%

Node based							
Concordant nodes			**Discordant nodes**		**Total**	**Discordant nodes**	**Discordant nodes %**
H+ M+		H- M-	H+ M-	H- M+			
Initial analysis	56	281	3	12	352	15	4.26%
Deeper levels	61	281	3	7	352	10	2.84%

Table 52: A summary of concordant and discordant nodes and cases when comparing Metasin with histology. H = histology, M = Metasin.

Initial analysis is the first histological result before further levels were carried out.

The table above shows there were 10 discordant cases and 15 discordant nodes before further levels were carried out. After deeper levels, the number of discordant cases fell to 6 and the number of discordant nodes fell to 10. The discordance rates of 3.89% and 2.84% for cases and nodes respectively is below the proposed accepted rate of discordance that GeneSearch set of 4%.

A similar table is also presented comparing GeneSearch and histology
A summary of the number of nodes and cases found to be concordant and discordant with Veridex and histology is tabulated. The discordance rate is also shown.

	Case based						
	Concordant cases		**Discordant cases**		**Total**	**Discordant cases**	**Discordant cases %**
	H+ G+	H- G-	H+ G-	H- G+			
Initial analysis	33	114	1	6	154	7	4.50%
Deeper levels	36	111	4	3	154	7	4.50%

	Node based						
	Concordant nodes		**Discordant nodes**		**Total**	**Discordant nodes**	**Discordant nodes %**
	H+ G+	H- G-	H+ G-	H- G+			
Initial analysis	57	282	2	11	352	13	3.70%
Deeper levels	60	279	5	8	352	13	3.70%

Table 53: A summary of concordant and discordant nodes and cases when comparing histology with GeneSearch. G = GeneSearch, H = histology. Initial analysis is the first histological result before further levels were carried out.

The table above shows there were 7 discordant cases and 13 discordant nodes before further levels were carried out. After deeper levels, the number of discordant cases stayed at 7 and the number of discordant nodes stayed at 13. The discordance rate of 4.5% for cases was above the accepted rate set by GeneSearch. However, the discordance rate for nodes is 3.7% which is below the discordance rate set by GeneSearch.

The discordant cases has a significantly greater clinical consequence than the discordant nodes as a patient with a positive concordant node ie positive for both histology and Metasin, but with one or more discordant nodes is still labelled as a positive patient and therefore will still undergo axillary clearance. Therefore, analysis of discordant cases only will be described in detail.

The table below shows the discordant cases comparing the Cq values of all three markers and the histology results with the size of the metastasis for the histology positive nodes.

Patient	Specimen	GS CK19 Cq Value	Metasin CK19 Cq Value	GS MGB Cq Value	Metasin MGB Cq Value	GS	Metasin	Initial Histology Result	Histology after levels
1	08-12082B1	0	0	0	36.2	negative	negative	negative	
	08-12082B3	0	0	0	36.9	negative	negative	negative	
	08-12082C1	0	0	32.6	31.5	negative	positive	negative	Negative
	08-12082C3	0	0	35	34.2	negative	negative	negative	
2	08-16837B	20.5	0	0	0	positive	negative	positive	Macro
3	09-11238B	0	29.5	32.6	29.8	negative	positive	negative	Micro
4	09-15813B	0	0	0	30.7	negative	positive	negative	Micro
	09-15813C	0	0	0	0	negative	negative	negative	
5	10-1643A	30.6	29.5	0	0	negative	positive	negative	Micro
	10-1643B	35.5	0	0	0	negative	negative	negative	
	10-1643C	0	0	0	0	negative	negative	negative	
6	08-17635B1	34.8	0	0	0	negative	negative	negative	
	08-17635B2	29.9	0	31.7	36.2	positive	negative	negative	Negative

Table 54. GS = GeneSearch. All cases discordant for GeneSearch and Metasin are presented including the Cq values for Ck19 and MGB, the histology result before and after levels and the size of metastatic tumour deposit. The boxes highlighted in red are the discordant results

The table shows four cases where Metasin is positive and GeneSearch is negative. Three of those cases were shown to be positive on histology after deeper levels had been cut. Therefore, one case is negative for both histology and GeneSearch and considered a false positive result.

There are two cases where GeneSearch is positive and Metasin is negative. One of these cases showed a macrometastasis indicating a false negative result for Metasin. The other case has not been shown to be positive on histology. If taking GeneSearch as the gold standard, this would be a false negative result but if considering histology as the gold standard, this would be a concordant result for Metasin.

There is only one case where both GeneSearch and Metasin were negative and histology showed a macrometastasis, thus being a false negative for both assays.

In summary, when comparing Metasin to histology, there is one false positive result and 2 false negative results. When comparing Metasin with GeneSearch, there are 2 false negative results and 4 false positive results, 3 of which were in fact shown to be positive on histology.

The table below compares the results of both nodes and cases with Metasin and GeneSearch.

Node based discordance						
Concordant nodes		Discordant nodes		Total	Discordant nodes	Discordant nodes %
G+ M+	G- M-	G+ M-	G- M+			
64	280	4	4	352	8	2.27%
Case based discordance						
Concordant cases		Discordant cases		Total	Discordant cases	Discordant cases %
G+ M+	G- M-	G+ M-	G- M+			
37	111	2	4	154	6	3.89%

Table 55: Comparing the results of cases and nodes of Metasin (M) vs GeneSearch (G)

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Eight nodes were found to be discordant when comparing Metasin and GeneSearch results. Six of those nodes are from six discordant cases and two nodes are from one concordant case which had three other concordant positive nodes.

The tables below summarise the number of cases and nodes tested, comparing Metasin to GeneSearch and histology.

Metasin vs GeneSearch		Metasin vs Histology	
Node Based Analysis		**Node Based Analysis**	
Positive nodes	64 (18.2%)	Positive nodes	61 (17.3)
Negative nodes	280 (79%)	Negative nodes	282 (80%)
Discordant nodes	8 (2.3%)	Discordant nodes	9 (2.5%)
Total	352	Total	352
Case Based Analysis		**Case Based Analysis**	
Positive patients	37 (24%)	Positive patients	37 (24%)
Negative patients	111 (72 %)	Negative patients	111 (72%)
Discordant patients	6 (3.8%)	Discordant patients	6 (3.8%)
Total	154	Total	154

Table 56: Results of Metasin for cases and nodes compared with GeneSearch (left) and Histology (right)

Chapter 8   Validating the Metasin assay

Several tests were carried out to validate the Metasin assay. The reproducibility of the assay was tested by running the same samples on different days with different users. The products were also run on a gel to confirm the right products were being amplified. The Cq values of monoplexed and multiplexed markers were also compared to see if multiplexing had an effect on the accuracy of the results. The RNA concentrations of some of the positive samples were compared to their PBGD Cq values for both Metasin and GeneSearch. Finally, a dilution series was also run using three positive samples and the efficiency calculated.

8.1   Comparing Metasin Cq values of the same samples on different runs done on different days by different users to illustrate reproducibility of results

Out of 16 positive cases (33 nodes) that were run, three nodes in three different cases had discordant MGB results. Two of these cases had concordant positive CK19 and so the cases were still positive. The third known positive case (15131B) is positive for MGB with a Cq value of 30.8 and negative for CK19 with a Cq value of 31.6 by GeneSearch. The case was found to be positive on Metasin with a MGB Cq value of 31.7 on one day by one user and negative on Metasin with a MGB Cq value of 33.6 on the other day with the other user.

The full table is available in the appendix (pg 226).

The following histogram illustrates the difference between the set of results obtained by each user. CK19 showed the least difference in Cq value with the largest difference being 0.6. PBGD Cq value difference was seen to be 0.8. MGB had the greatest difference in Cq value with 1.9.
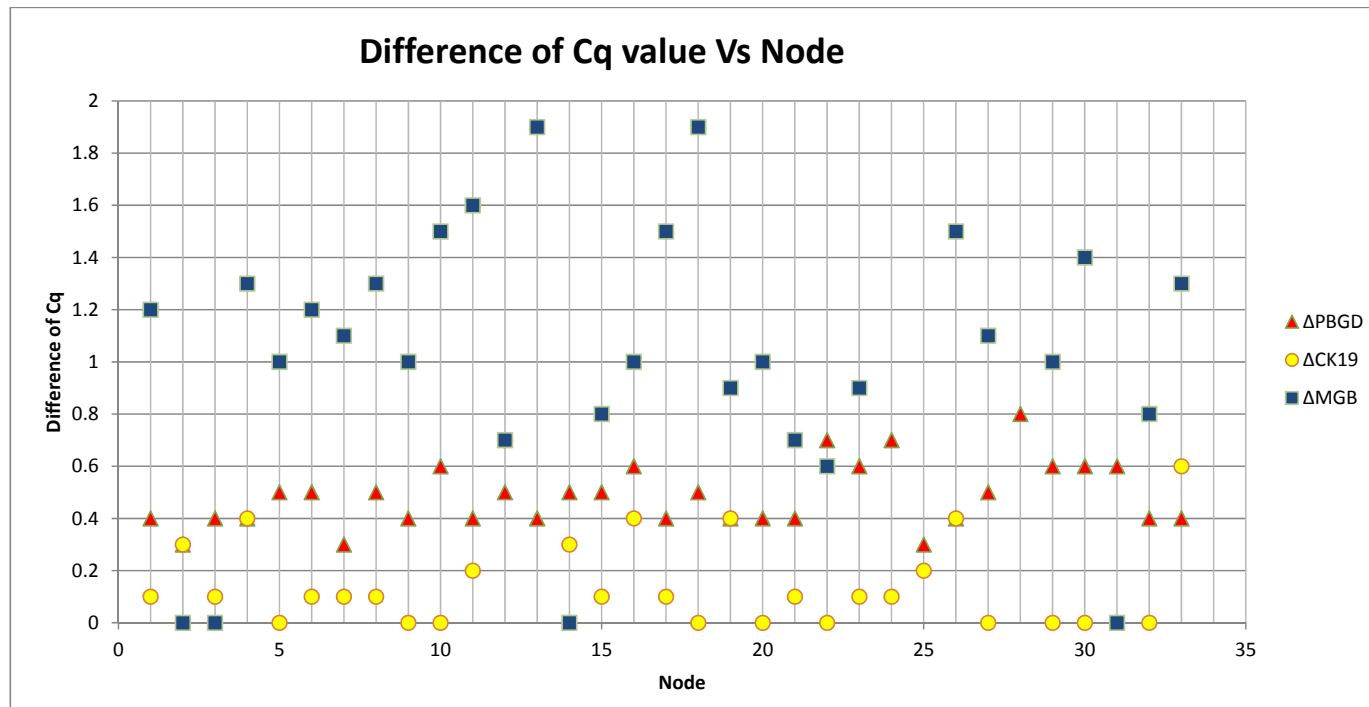
Graph 7. Comparison of Cq Values to assess reproducibility of the Metasin assay

8.2   RNA product identification

The PCR products were run on a gel to ensure the correct amplicons were being replicated.
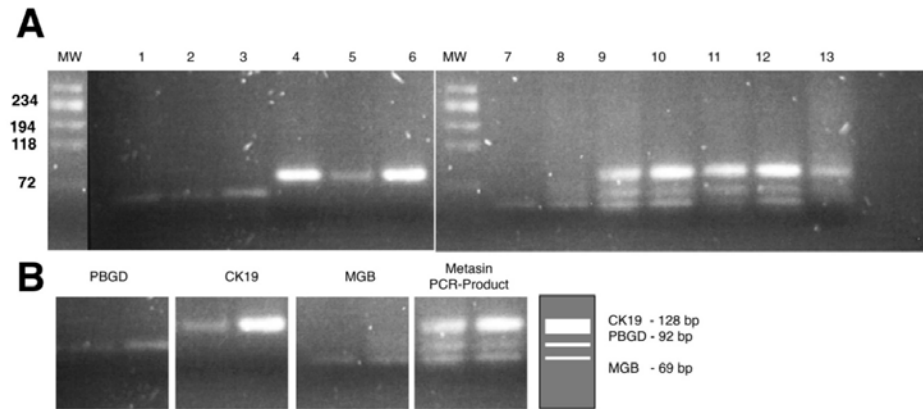
Figure 15 Gel electrophoresis of positive nodes that were monoplexed and triplexed

Figure A shows the fluorescence of the PCR products after amplification of PBGD (lanes 1,2 and 3), CK19 (lanes 4,5 and 6) and MGB (lanes 7 and 8). Lanes 9 to 13 show fluorescence of known positive nodes. The molecular weight marker (ΦXHae III marker) is shown on the left side and between lanes 6 and 7. The size markers correspond to CK19 (128 bp), PBGD (92bp) and MGB (69bp). Figure B shows the bands for each monoplexed marker and two triplexed samples more closely with a schematic on the right illustrating the expected band sizes corresponding to the three amplicons.

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8.3   RNA concentration and yield

The RNA concentrations of the samples are reflected in the PBGD Cq values and do not vary greatly. The ratio of A260/280 shows most samples around 2, indicating the RNA is pure with hardly any contamination of protein. The full table is available in the appendix (pg 228).

8.4   Comparing monoplexed and multiplexed Cq values

The table compares the Cq values of the monoplexed and multiplexed markers.

Sample	PBGD mono	PBGD multi	CK19 mono	CK19 multi	MGB mono	MGB multi
7867D	25.3	25.4	27.9	27.5	22.8	22.8
3256E	24.9	25.3	27.7	28.4	29.4	29
7638	22.7	23.3	16.3	16.6	16.3	19.4
6173B	25.2	25.7	20.3	20.3	20.8	22.9

Table 57 Comparing the Cq values of monoplexed and multiplexed markers.

The results in these samples correlate closely.

8.5   The no-RT step run

A control run was done to check the mastermix that was to be used in the no RT run would work.

Sample	PBGD	CK19	MGB
17502B2 (positive control)	25.9	18.5	26.4
18609B (negative control)	27.3	0	0

Table 58.1 Confirming the mastermix works with Cq values for the positive and negative control.

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The assay was then run without the RT step.

Sample	PBGD
7867D	0
10762D	0
3256E	0
11531C1	0
10931B	0
745C	0
925B	0
17502B2	0
12439B	0
16837B	0
2344C	0
2487B	0
12043B	0

Table 58.2 The no RT step run results

The table above shows no detection of PBGD and therefore no genomic DNA was shown to be present.

8.6   Dilution series and PCR efficiency

The RNA of three samples were diluted tenfold upto four times and run to give the efficiency of the PCR. The Cq values of all three samples are also given.

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Results

Well	Exclude	Sample Name	Assay Name	Assay Role	Cq	Cq Mean	Std. Dev. Cq	Quantity	Mean Qty.	Std. Dev. Quantity	Tm1	Tm2	Tm3
A1		cDNA	Assay 1	Standard	18.56	18.69	0.18	1000000	1000000	0	82.6	85	N/A
A3		cDNA	Assay 1	Standard	23.03	22.88	0.21	40000	40000	0	83.5	87.1	75.1
A4		cDNA	Assay 1	Standard	25.41	25.45	0.1	8000	8000	0	83.2	88	75.1
B2		cDNA	Assay 1	Standard	20.53	20.62	0.12	200000	200000	0	83.8	86.5	72.4
B3		cDNA	Assay 1	Standard	22.73	22.88	0.21	40000	40000	0	83.5	86.2	N/A
B4		cDNA	Assay 1	Standard	25.38	25.45	0.1	8000	8000	0	83.5	87.1	N/A
B5		cDNA	Assay 1	Standard	28.02	28.09	0.1	1600	1600	0	83.5	75.1	N/A
C1		cDNA	Assay 1	Standard	18.81	18.69	0.18	1000000	1000000	0	84.1	86.8	N/A
C2		cDNA	Assay 1	Standard	20.7	20.62	0.12	200000	200000	0	83.8	86.5	N/A
D4		cDNA	Assay 1	Standard	25.56	25.45	0.1	8000	8000	0	83.8	86.8	75.7
D5		cDNA	Assay 1	Standard	28.17	28.09	0.1	1600	1600	0	83.8	75.1	N/A

Table 59. Cq values in the dilution series
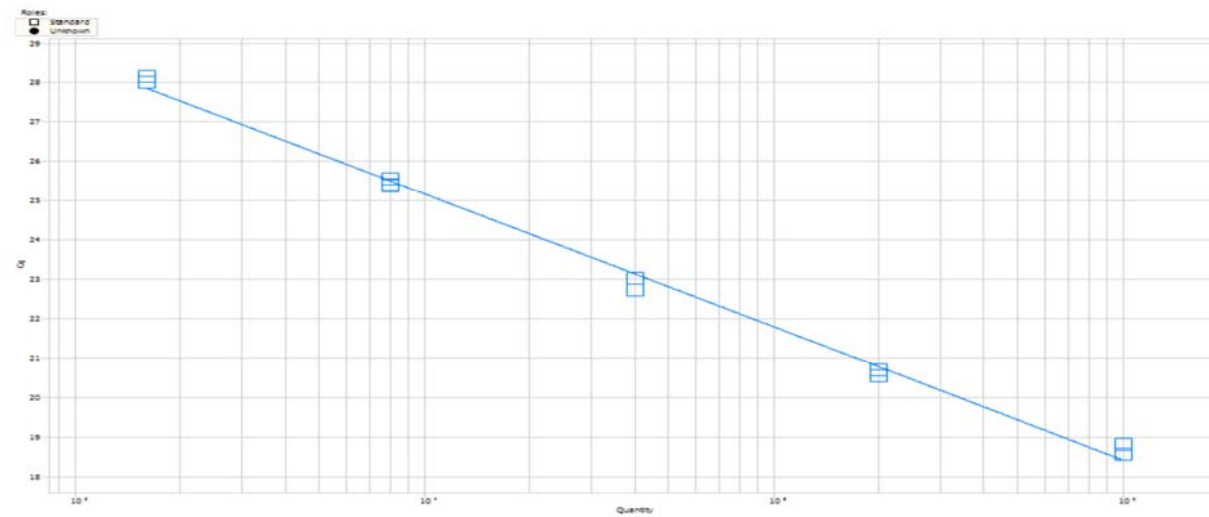
Standard Curve

Graph 8. Standard curve and PCR efficiency of Metasin

The efficiency is close to 100% (97.65%).

In summary, the PCR amplification was shown to produce the correct amplicons at the end of the reaction as evidenced by the expected bands seen when the PCR products were run on a gel. The concentration of the RNA products did not vary widely and the RNA was found to be pure with the ratio of A260/280 approximately 2. Cq values of monoplexed and multiplexed markers correlated closely with no discordant results. The no-RT experiment showed no detectable PBGD. PCR efficiency was found to be almost 100%. When the assay was run on different days by different users, three nodes were discordant for MGB and one case was discordant highlighting the need for improvement in the reproducibility of the assay.

Chapter 9

The purpose of the MD was to set-up and validate an intra-operative real-time RT-qPCR assay to detect metastatic breast cancer metastases in sentinel lymph nodes. The aim was to be able to replace the commercial assay GeneSearch (Veridex) which was about to be withdrawn.

Molecular pathology and diagnostics has a major impact on the diagnosis and treatment of patients with cancer. Real-time RT-qPCR is just one of many tools used by researchers, clinicians and technicians to diagnose various genetic forms of cancer. It is highly specific and sensitive, relying on appropriate primers which are specific to the target of interest.

The Metasin assay was initially optimised using FAM-labelled hydrolysis probes on the LC480 platform. The clinical requirement of the Metasin assay is that the time taken to reach a result should not take longer than approximately 26 minutes, emulating the run time of GeneSearch. In order to achieve this, the LC480 was replaced with the SmartCycler and the primers and probes multiplexed in order to improve the speed of the Metasin assay.

The table below summarises the steps taken for the LC480 and Metasin, including the methods employed in reducing the run time.

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LC480	Metasin
Primer concentration	Primer concentration
Probe concentration	RNA volume
RNA volume	
Reducing the run time	**Reducing the run time**
Annealing step	Annealing step
	Denaturing step
	Reverse transcription step

Table 60. The optimising steps carried out on both platforms

Once Metasin was optimised, 154 cases having 352 nodes were tested and their Cq values compared to those of GeneSearch. Out of 154 cases, 111 were found to be negative and 37 were found to be positive for both assays. Six cases were discordant. The sensitivity and specificity of Metasin were found to be 95% and 98% respectively. The sensitivity and specificity of GeneSearch were found to be 90% and 100% respectively. The error rate of Metasin was 3.89% compared to GeneSearch with 4.50%.

This chapter aims to discuss unexpected findings encountered during the validation process with a detailed discussion of the the discordant results. The effect the Metasin assay will have on patients and necessary further work are described.

9.1   Validating the Metasin assay

Several tests were taken to validate the assay. The RNA quantity was checked using a spectrophotometer. However, a further check for RNA quality was not performed and should be carried out to gain further reassurance regarding the integrity of the RNA extracted. The desired amplicons that were targeted during PCR were found to be present in the PCR product when tested using gel electrophoresis. When the RT step was omitted from the PCR run, no genomic DNA was detected. Comparing the results

of the monoplexed and multiplexed markers showed no overall significant differences in Cq values for the markers. PCR efficiency was found to be nearly 100% at 97.65%.

The reproducibility test highlighted flaws in the assay which need to be addressed. The runs carried out to investigate reproducibility of results illustrate the importance of having a standardised assay which would help reduce the chance of user error. For example, large batches of pre-prepared primer and probe mixes could be tested on several known positive and negative samples and then stored before being used in a diagnostic run. Adequate training of users of the assay is crucial to minimise errors made by inexperienced users. Veteran users of the assay should train and supervise new users to check there are no bad practices or errors made or learnt which could be passed to others. A standard operating procedure (SOP) has already been drafted for the Metasin assay and is available on page 240.

9.2 The discordant cases

The majority of cases were concordant with Metasin and GeneSearch. Out of 154 cases, there were 6 discordants, 3.9% of the total number of cases.

9.2.1 Troubleshooting discordant cases

In investigating the discordant results, an organised and logical trouble-shooting approach was developed. Different problem scenarios which came up during the optimising and validation process were addressed. Where appropriate, cases which fall into those scenarios are included. As noted earlier, the discordance rate of Metasin is 3.89% and the cases described below form a minority of the overall cases tested.

Problem:

- The positive and/or negative control did not work and/or the PBGD Cq value for any node did not work

Possible solution:

- Problem with the primer/probe mix
- Problem with the Mastermix
- No RNA was added

If the nodes to be investigated registered a PBGD Cq value but the control/controls did not, then fresh RNA was extracted from a known positive and negative node and using the same primer/probe mix the assay was re-run. If the controls worked but no PBGD Cq value was obtained for the unknown nodes, fresh RNA was extracted and the assay repeated with the same primer/probe mix.

In my experience, this would usually solve the problem of no PBGD in some nodes in the same assay.

If no nodes were positive for PBGD, a fresh batch of primer/probe mix would be made up. Fresh Mastermix is always made up for new runs.

Problem:

- The controls worked, however, there is discordance

Possible solution:

1. Discordance with histology

Metasin is negative and histology is positive

New primer/probe mix was made up and fresh RNA extracted. If the result was positive then it was considered a user error. If the result was still negative, this could be taken as either a sampling error or a false negative result.

Metasin is positive and histology is negative.

The blocks of the discordant node are pulled out and upto 10 extra levels were cut and prepared for microscopic assessment along with immunohistochemistry for a pancytokeratin MNF116 and/or CK19. If the extra levels revealed metastatic tumour, Metasin was vindicated. There were three cases out of 154 where the initial histology result was negative, however, further levels and immunohistochemistry revealed micrometastases (please see tables 52 and 54). If the extra levels did not reveal metastasis, this could be taken as a sampling error or a false positive result.

2. Discordance with GeneSearch

Metasin is positive and GeneSearch is negative

If histology was positive, Metasin was correct and this was a case missed by GeneSearch. The GeneSearch assay was re-run for the node a second time to try to rule out user error.

If histology was negative, extra levels were cut to try and determine the presence of metastases. If metastases were found, then Metasin was correct. If not, then the Metasin assay was re-run using fresh primer/probe mix and fresh extracted RNA. If the node was negative the second time round, the first false positive result was considered a user error or a contamination problem. If despite mixing a fresh batch of primer/probe mix which worked on the controls and extracting fresh RNA from the discordant node, the result was still positive, either there was still a contamination issue or it was a sampling error and a false negative GeneSearch result. One case out of the six discordant cases previously described in section 7.1 was positive for MGB only with a Cq value of 31.5 raising the possibility of a micrometastasis in the slice given to Metasin with none of the focus seen on the slide. The GeneSearch assay did detect MGB with a Cq value of 32.6 which was 1.6 away from a positive result.

Metasin is negative and GeneSearch is positive

If histology was negative, further levels and immunohistochemistry was done to try and confirm the node was positive on histology. If it remained negative, the GeneSearch assay was re-run for the node. If the second GeneSearch result was negative, then the first erroneous result was most likely due to user error or contamination. If it remained positive, this may be a false-positive result or Metasin may be giving a false-negative result and there may be sampling error.

If histology is positive, then a fresh primer/probe mix was made, fresh RNA extracted and the Metasin assay run again. If the node was then positive, it was put as a user error. If the node remained negative for Metasin, the node was run again on GeneSearch to confirm it was positive. Despite repeated attempts at running the node on Metasin on different days with different operators, if it still remained negative, the same RNA used on Metasin was run on GeneSearch to test the RNA. In unresolved

cases, the PCR products from Metasin could be run on a gel to try and identify the three products PBGD, CK19 and MGB. If the products are visible on the gel, it suggests a probe problem where the product was present but could not be detected. If the products are not detected, it suggests a primer problem. The last step would be to consider sequencing the PCR products to detect the presence of PBGD, CK19 and MGB. Two out of the six discordant cases fall into this category. One case in which there was a macrometastasis on histology was thought to be possibly due to RNA degradation as the sample was constantly thawed and frozen over a two year period. The other case was histology negative where GeneSearch picked up CK19 with a Cq value of 29.9, just 0.2 away from a negative result. Metasin did not detect any CK19.

Commented [SP3]: You were asked to expand this and include a comment on the likelihood of these various scenarios. At present it still implies that these are frequent occurrences. Please amend and add an additional paragraph.

9.2.2   Discordance between Metasin and histology

Histology will remain the gold standard for assessment of the sentinel lymph node and therefore submitting the whole node to Metasin is a step that may be too rash for most histopathologists who feel that histological evidence of metastasis is needed to constantly validate the Metasin assay findings. The drawback is the chance of sampling discordance whereby if a micrometastsis is present in the 2mm slice given to either test, a discrepancy will almost certainly arise.

For the purposes of the assay, the view was taken that even if Metasin or histology did not detect a positive node, but the case overall was found to be positive with other nodes, then that was a concordant result. The majority of nodes and therefore cases were concordant. However, there were 6 cases that were found to be discordant for Metasin and histology, with 4 cases positive for Metasin and negative for histology and 2 cases negative for Metasin and positive for histology.

The table illustrates the possible outcomes and the possible reasons behind the discordance between the molecular assays and histology (Douglas-Jones and Wood, 2009).

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Molecular assay (Metasin and GeneSearch)	Histology	Interpretation
Positive	Positive	Concordance
Positive	Negative	? False positive ? Sampling error
Negative	Positive	? Sampling error
Negative	Negative	Concordance

Table 61: Showing the possible reasons for discordance

Discordance between histology and both molecular assays is most likely due to sampling. Alternate slices of the node are given to the assays and histology resulting in unavoidable sampling discordance. An example of a case that highlights this point is the case where one node which was found to be positive with histology but negative with both Metasin and GeneSearch.

A closer analysis was done of this node. The node was sliced into five slices and slices 1, 3 and 5 were given to the molecular assays and slices 2 and 4 to histology. For the histological assessment of a node, each slice is examined on three levels: L1, L2 and L3. In this particular node, the first level in slice 2 abutts the deep surface of slice 1. The second level in slice 2 was taken 150µm deep and the third level another 150µm deep. On histological examination of slice 2, levels 1 and 2 showed a macrometastatic tumour deposit measuring 3mm.
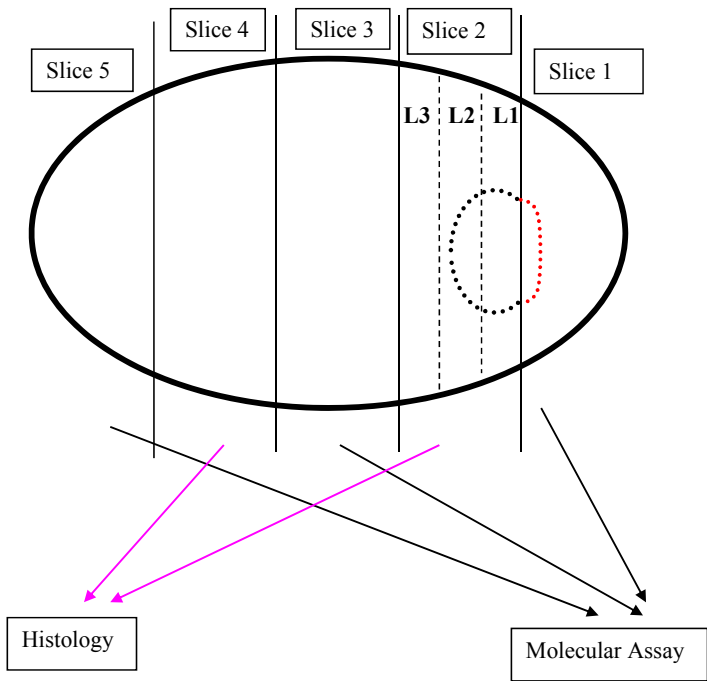
Figure 16. Diagram of the slices and the location of the macrometastasis

The red dotted line indicates the unknown area.
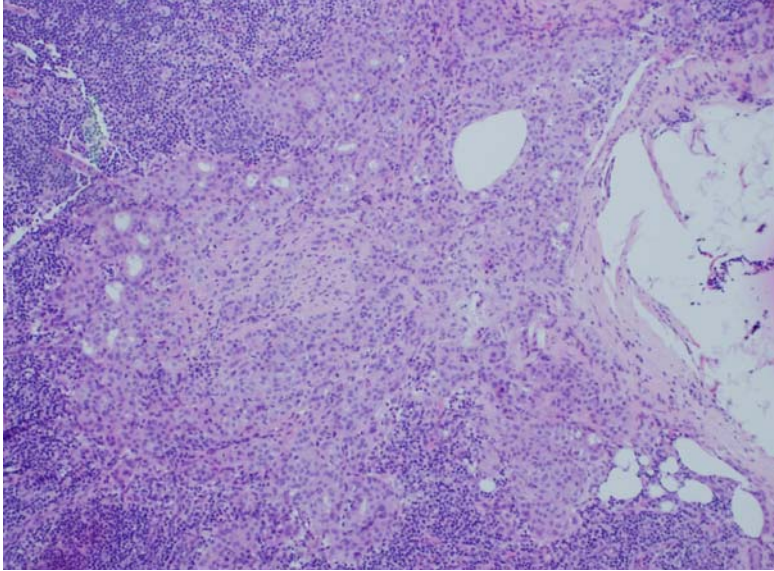
Figure 17. Level 2 of slice 2 showing a 3mm macrometastasis (x10).

The metastatic tumour deposit was seen in slice 2 only and in L1 and L2 but not L3. This indicates that the cancer did not spread through slice 2 to reach slice 3 and therefore the assays would have found slices 3 and 5 negative. However, L1 in slice 2 was positive and so the deep surface of slice 1 was likely to be positive for metastatic cancer.
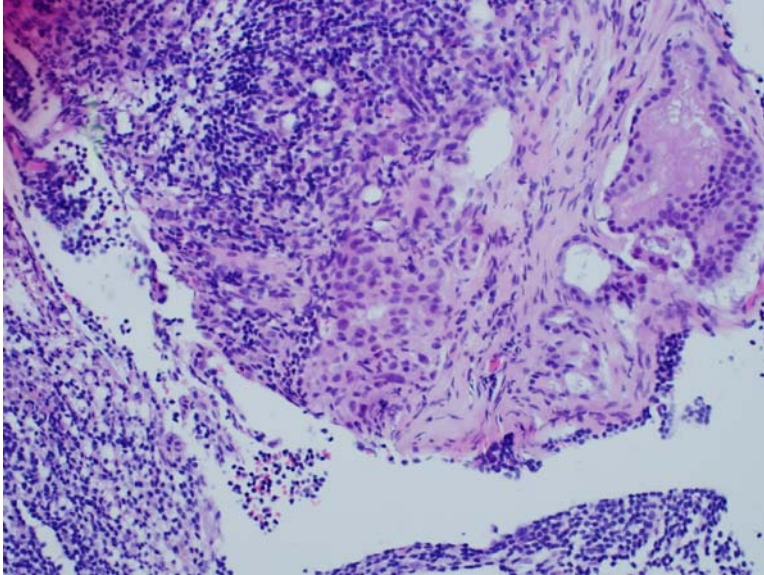
Figure 18. Level 1 of slice 2 showing part of the macrometastasis (x20). This level is the closest to slice 1.

After analysing the histology results, the sample was run twice more on GeneSearch and three times on the LC480 and Metasin and the same results obtained. Therefore, this case represented a false negative for all three molecular assays.

It would be unlikely for all three assays to be unsuccessful in picking up a macrometastasis. Therefore, the likeliest explanation is that the slice with the cancer may have been thicker than 2mm and contain the whole focus of tumour. The lymph node was measured to be 14mm in maximum length, which should result in 7 slices if the node was cut into 2mm slices. However, there were only 5 slices cut. This supports the idea that the slices were too thick and raises the possibility of the whole tumour deposit being in one slice that was given to histology.

Despite the discordant cases, the error rate of Metasin of 3.89% falls below the error rate acceptable for the commercially available GeneSearch assay.

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9.3 Impact of the assay on the patient and the Trust

The validation results were found to be accurate enough for the Metasin to be first run alongside GeneSearch and then to be used as a diagnostic assay. The effect the assay will have on the patient and the Trust has been described in detail in the introduction. It will avoid an unnecessary second operation for the patient, reducing their anxiety and helping with their recovery. A reduction in second operations will also reduce the pressures on the Trust and the NHS in terms of the beds needed, operating time and theatres used. However, it should be kept in mind there may be other reasons for patients to undergo second operations, for example, in situations where a wide local excision has been found to have a positive margin.

Metasin is cost-effective, a huge advantage in times of financial constraints and cost-cutting in the NHS. It costs approximately £50 to test a sentinel lymph node using the Metasin assay. The average cost of a completion axillary clearance is estimated at £2227, a huge saving for the Trust (Nadi et al, 2012).

Importantly, Metasin has also been shown to be more sensitive and specific in picking up metastases compared to conventional intraoperative techniques of frozen section and TIC, improving the detection rate.

9.4 Future work

The concept of detecting metastatic breast cancer in sentinel lymph nodes using the markers CK19 and MGB has been trialled and tested by various institutions and shows the GeneSearch assay is highly specific and sensitive. Therefore, the concept of the Metasin assay which uses the same principle was already known and accepted. For Metasin to replace GeneSearch it would need to show the same level of performance, if not better.

During the optimisation process, a number of decisions were made regarding primer and probe concentrations and RNA volume used in the assay. These decisions were influenced by performance and to allow for easy calculations and preparation of the PCR mixture but also by cost and time restraints. In order to deliver a functioning assay at a specified time and within budget, certain procedures were not carried out. When optimising the assay, triplicate runs could have been run for all samples to ensure accurate results. Primer and probe sets could have been made into batches and tested on positive controls before being used in optimising runs instead of being used straightaway in runs. Discordant cases could have had sequencing of DNA to investigate the causes of discordance. The overall approach taken was largely pragmatic and due to the increased cost that would incur.

There are further improvements that could be made. For example, instead of having a single reference gene, a panel of reference markers could be used in order to improve the reliability and accuracy of the assay. More samples would also need to be run to make the assay more statistically significant. Only then can the Cq cut-off values be re-examined to ensure accuracy. RNA samples from a further 193 anonymised nodes were tested to assess the validity of the assay. All 104 negative nodes were found to be negative and all nodes with macro-metastases were found to be positive with Metasin. However, 15 out of 33 nodes with micrometastatses were found to be negative by Metasin with Cq values ranging from 0.1 to 1.1 from the cut-offs (Al-Ramadhani et al, 2013). This raises the need for standardisation of the assay.

However, there are a growing number of institutions that advocate sparing the patient with micrometastases an axillary clearance although this is not entirely accepted. Giuliano et al reported after the ZOO11 trial that patients with a positive sentinel lymph node who match their inclusion criteria should be spared axillary clearance as there was no additional benefit to overall survival (discussed further in section 1.2.1, pg 37).

Commented [SP4]: You were asked to add a more detailed description of th Z11 study, which you have not done. Please amend and do this.

Contamination is also an important issue when using PCR. Ideally, the preparation stage and PCR stage should be done in separate rooms with a one-way sample flow. Other measures that would help are frequent glove changes, dedicated pipettes and experienced and trained laboratory staff. Currently, the Metasin assay is being used successfully as a diagnostic assay. A SOP has been produced which will enable the assay to become standardised and therefore limit the chances of error. The primer/ probe mixes can also be made either in large batches or by an external expert provider that can quality assure its product and provide assistance or advice when unexpected results occur.

Appendix 1 Materials and Method

1.1   Full list of Materials

The following list of equipment, reagents and primers and probes were used during the optimisation and validation of the Metasin assay.

COMPANY	PRODUCT NAME	CATALOGUE NUMBER
ROCHE	LIGHTCYCLER 480 RNA HYDROLYSIS PROBES	4991885001
QIAGEN	RNEASY MINI KIT (50)	74104
QIAGEN	BUFFER RLT (220ml)	79216
CEPHEID	CEPHEID 25 µL REACTION TUBES (for SmartCycler) 50 pcs/bag	900-0003
QIAGEN	VAC CONNECTORS 100/bag	19407
QIAGEN	VAC VALVES 24/pk	19408
OMNI INTERNATIONAL	OMNI TIPS DISPOSABLE PROBES for Omni Homogeniser 25pk 7mmx110mm	30750
VWR	8mL PLASTIC CULTURE TUBES for homogenisation 13x100mm 125/bag	211.0074
VWR	15mL POLYSTYRENE TUBES for homogenisation 17x120mm 500/bag	734-0450
VWR	15ML CENTRIFUGE TUBE WITH FLAT CAP 500/BAG	525-0400
FISHER BRAND	SAV-IT CLOSURES 12mm/13mm GREEN PK OF 100	02-707-10
BECTOR DICKINSON	50mL FALCON BLUEMAX POLPROPYLENE CONICAL TUBE for 70% Ethanol 25/pk	352098
(TRADE WINDS DIRECT)	DISPOSABLE FORCEPS (autoclavable) 100/pk	DF8088N

FISHER BRAND	WEIGHING DISH 89X89X25mm 500PCS/BOX	08-732-113
SWANN-MORTON	DISPOSABLE SCALPELS (10/pk)	SCA-310-030K
FISHER BIOREAGENTS	WATER STERILE DEPC TREATED (NUCLEASE FREE) 100mL	BPE2484-100
SWANN-MORTON	MICROCENTRIFUGE TUBE SNAP TOP graduated 1.5mL 500/pk	FB56089
FISHER SCIENTIFIC	PIPETTOR TIP SUREONE FILTER STERILE RACK MICROPOINT TIP 0.1 - 10µl graduated 960 tips/pk	FB78100
FISHER SCIENTIFIC	PIPETTOR TIP SUREONE FILTER STERILE RACK BEVELLED TIP 10-100µL 960 tips/pk	FB78108
FISHER	PIPETTOR TIP SUREONE FILTER STERILE RACK MICROPOINT TIP graduated 100-1000µl 960 tips/pk	FB78112
FISHER	PIPETTOR TIP FINNTIP STERILE 2-10ML 24 tips/pk	PMP-103-260E
VWR	PIPETTE TIP BIOHIT SAFETYSPACE FILTER TIP 2-100/120µl 960 tips/pk	790101F
CALBIOCHEM	β-MERCAPTOETHANOL 250ml	44203
SIGMA ALDRICH	200-PROOF (ABSOLUTE) ETHANOL, molecular biology grade	245119-1L
FISHER	MICROCENTRIFUGE TUBES, 1.5ML WITH SCREW CAP & O-RING ATTACHER, AMBER	TUL-918-096B
VWR	SUPERSPIN TUBES, 1.5ML YELLOW TUBES	211-0017
VWR	SUPERSPIN TUBES, 1.5ML ORANGE TUBES	211-0018
VWR	SUPERSPIN TUBES, 1.5ML PURPLE TUBES	211-0020
VWR	SUPERSPIN TUBES, 1.5ML BLUE TUBES	211-0021
ROCHE	LIGHTCYCLER 480 SEALING FOIL, 50 FOILS	

		04729757001
ROCHE	LIGHTCYCLER 480 MULTIWELL PLATE (96)	04729692001
ROCHE	LIGHTCYCLER COLOUR COMPENSATION SET (FOR 5 CALIBRATION REACTIONS)	12158850001
ROCHE	LIGHTCYCLER MULTICOLOUR DEMO SET (5 COLOUR COMPENSATION RUNS)	03624854001
QIAGEN	QUANTITECT REVERSE TRANSCRIPTION KIT, 200 X 20 ULS REACTIONS	205313
ALPHA LAB	0.1-10UL CLEAR TIP STERILE PIPETTE TIPS (96/PACK)	LW1136
ALPHA LAB	1-200UL CLEAR TIP STERILE PIPETTE TIPS (96/PACK)	LW6365S
ALDRICH	MICROFUGE TUBE 1.5ML, SAFE-LOCK, 1000 TUBES	7555874
ALDRICH	MICROFUGE TUBE 0.5ML	Z606332-500EA
JENCONS	BIOHIT FILTER TIPS, 0.1UL-10UL, 96 TIPS X 10	613-0857
VERIDEX	GENESEARCH BREAST LYMPH NODE (BLN) TEST KIT	2900004
VERIDEX	GENESEARCH RNA SAMPLE PREPARATION KIT	2900005
COSTAR	MICROCENTRIFUGE TUBE 1.7MI 500 TUBES/BAG	3621
BIOLABS	ØX174 DNA-HAEIII DIGEST	N3026S
FLUKA ANALYTICAL	AGAROSE	05066-50G
SIGMA	TBA BUFFER	

Synthesis Scale: 0.010 7mol

Condition: 10 nmol lyo

Purification: GSF

CK19 F2 (FORWARD PRIMER)

CAgCCACTACTACACgACCATC   LENGTH 22MER

CK19 R2 (REVERSE PRIMER)

CAAACTTggTTCggAAgTCATC   LENGTH 22MER

SCGB2A2_S (FORWARD PRIMER)

CTCCCAgCACTgCTACgC   LENGTH 18MER

SCGB2A2_b (REVERSE PRIMER)

GgATTgATTgTCTTggAAATCACA   LENGTH 24MER

PBGD_F (FORWARD PRIMER)

TgTggTgggAACCAgCTC   LENGTH 18MER

PBGD_R (REVERSE PRIMER)

TgTTgAggTTTCCCCgAAT   LENGTH 19MER

PROBES

PBGD (NM_000190)

ctcctgaactccagatgcggga

CK19 (NM_002276)

192

cagccagacgggcattgtcg

MGB (NM_002411)

Ctctggctgccccttattggag

1.2 Genetic sequences for the markers PBGD, CK19 and MGB

The following are the gene sequences for the markers used in the assay. The bold red sequences are from the UPL website. The underlined sequences and the black bold letters are the primer sequences designed by TIB MOLBIOL. The blue sequences are the probes from TIB MOLBIOL. The probe sequences from the UPL website are not available to the public.

PBGD mRNA (NM_000190)

```
   1 agcaggtcct actatcgcct ccctctagtc tctgcttctt tggatccctg aggagggcag
  61 aaggaagaaa acagcccaaa gatgagagtg attcgcgtgg gtacccgcaa gagccagctt
 121 gctcgcatac agacggacag tgtggtggca acattgaaag cctcgtaccc tggcctgcag
 181 tttgaaatca ttgctatgtc caccacaggg gacaagattc ttgatactgc actctctaag
 241 attggagaga aaagcctgtt taccaaggag cttgaacatg ccctggagaa gaatgaagtg
 301 gacctggttg ttcactcctt gaaggacctg cccactgtgc ttcctcctgg cttcaccatc
 361 ggagccatct gcaagcggga aaaccctcat gatgctgttg tctttcaccc aaaatttgtt
 421 gggaagaccc tagaaaccct gccagagaag ag**tgtggtgg gaaccagctc** cctgcgaaga
 481 gcagcccagc tgcagagaaa gt**tcccgcat ctggagttca ggag**tattcg gggaaacctc
 541 **aaca**cccggc ttcggaagct ggacgagcag caggagttca gtgccatcat cctagcaaca
 601 gctggcctgc agcgcatggg ctggcacaac cgggttgggc agatcctgca ccctgaggaa
 661 tgcatgtatg ctgtgggcca gggggccttg ggcgtggaag tgcgagccaa ggaccaggac
 721 atcttggatc tggtgggtgt gctgcacgat cccgagactc tgcttcgctg catcgctgaa
 781 agggccttcc tgaggcacct ggaaggaggc tgcagtgtgc cagtagccgt gcatacagct
 841 atgaaggatg ggcaactgta cctgactgga ggagtctgga gtctagacgg ctcagatagc
 901 atacaagaga ccatgcaggc taccatccat gtccctgccc agcatgaagat ggccctgagg
 961 atgacccaca gttggtaggc atcactgctc gtaacattcc acgagggccc cagttggctg
1021 cccagaactt gggcatcagc ctggccaact tgttgctgag caaaggagcc aaaaccatcc
1081 tggatgttgc acggcagctt aacgatgccc attaactggt ttgtggggca cagatgcctg
```

1141 ggttgctgct gtccagtgcc tacatcccgg gcctcagtgc cccattctca ctgctatctg

1201 gggagtgatt accccgggag actgaactgc agggttcaag ccttccaggg atttgcctca

1261 ccttggggcc ttgatgactg ccttgcctcc tcagtatgtg ggggcttcat ctctttagag

1321 aagtccaagc aacagccttt gaatgtaacc aatcctacta ataaaccagt tctgaaggt

CK19 mRNA (NM_002276)

   1 agatatccgc ccctgacacc attcctccct tcccccctcc accggccgcg ggcataaaag

  61 gcgccaggtg agggcctcgc cgctcctccc gcgaatcgca gcttctgaga ccagggttgc

 121 tccgtccgtg ctccgcctcg ccatgacttc ctacagctat cgccagtcgt cggccacgtc

 181 gtccttcgga ggcctgggcg gcggctccgt gcgtttttggg ccggggggtcg cctttcgcgc

 241 gcccagcatt cacggggggct ccggcggccg cggcgtatcc gtgtcctccg cccgctttgt

 301 gtcctcgtcc tcctcggggg cctacggcgg cggctacggc ggcgtcctga ccgcgtccga

 361 cgggctgctg gcgggcaacg agaagctaac catgcagaac ctcaacgacc gcctggcctc

 421 ctacctggac aaggtgcgcg ccctggaggc ggccaacggc gagctagagg tgaagatccg

 481 cgactggtac cagaagcagg ggcctgggcc ctcccgcgac **taca****gccact actacacgac**

 541 **catc**aggac ctgcgggaca agattcttgg tgccaccatt gagaactcca ggattgtcct

 601 gcagat**cgac aatgcccgtc tggctg**ca**ga tgacttccga accaa (exon3)gtttg** agacggaaca

 661 ggctctgcgc atgagcgtgg aggccgacat caacggcctg cgcagggtgc tggatgagct

 721 gaccctggcc aggaccgacc tggagatgca gatcgaaggc ctgaaggaag agctggccta

 781 cctgaagaag aaccatgagg aggaaatcag tacgctgagg ggccaagtgg gaggccaggt

 841 cagtgtggag gtggattccg ctccggggcac cgatctcgcc aagatcctga gtgacatgcg

 901 aagccaatat gaggtcatgg ccgagcagaa ccggaaggat gctgaagcct ggttcaccag

 961 ccggactgaa gaattgaacc gggaggtcgc tggccacacg gagcagctcc agatgagcag

1021 gtccgaggtt actgacctgc ggcgcaccct tcagggtctt gagattgagc tgcagtcaca

1081 gctgagcatg aaagctgcct tggaagacac actggcagaa acggaggcgc gctttggagc

1141 ccagctggcg catatccagg cgctgatcag cggtattgaa gcccagctgg gcgatgtgcg

1201 agctgatagt gagcggcaga atcaggagta ccagcggctc atggacatca agtcgcggct

1261 ggagcaggag attgccacct accgcagcct gctcgaggga caggaagatc actacaacaa

194

1321 tttgtctgcc tccaaggtcc tctgaggcag caggctctgg ggcttctgct gtcctttgga

1381 gggtgtcttc tgggtagagg gatgggaagg aagggaccct taccccggc tcttctcctg

1441 acctgccaat aaaaatttat ggtccaaggg aaaaaaaaa aaaaaaaaa

MGB mRNA (NM_002411)

   1 acagcggctt ccttgatcct tgccacccgc gactgaacac cgacagcagc agcctcacca

  61 tgaagttgct gatggtcctc atgctggcgg ccct**ctccca gcactgctac gc**ag (exon 2)g**ctctg**

121 **gctgcccctt attggag**aa<u>t **gtga**tttcca agacaatcaa tccaca</u>agtg tctaagactg

181 aatacaaaga acttcttcaa gagttcatag acgacaatgc cactacaaat gccatagatg

241 aattgaagga atgttttctt aaccaaacgg atgaaactct gagcaatgtt gaggtgttta

301 tgcaattaat atatgacagc agtctttgtg atttattta actttctgca agacctttgg

361 ctcacagaac tgcagggtat ggtgagaaac caactacgga ttgctgcaaa ccacaccttc

421 tctttcttat gtctttttac tacaaactac aagacaattg ttgaaacctg ctatacatgt

481 ttattttaat aaattgatgg ca

1.3 Tables showing the volumes of homogenization buffer and the volume to be transferred to the spin column during RNA extraction and purification

1.3.1   Volume of homogenization buffer required according to tissue weight

Tissue weight (mg)	Homogenization buffer (mL)
3 – 149	2
150 – 199	3
200 – 249	4
250 – 299	5
300 – 349	6
350 – 399	7
400 – 449	8
450 – 499	9
500 – 550	10
Greater than 550	To be divided further

(GeneSearch test kit insert)

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1.3.2   Volume of homogenate-ethanol mix to transfer to the spin column

Tissue weight (mg)	Volume of homogenate-ethanol mix (µl)
3 – 39	700
40 – 49	500
50 – 59	400
60 – 69	350
70 – 79	300
80 – 89	250
90 – 99	225
Greater than or equal to 100	200

Appendix 2 Results

2.1   Optimisation results – changing RNA template volume

For an RNA volume of **2μl**, the following Cq values were obtained.

Sample	Marker	Cq value	GeneSearch Cq value
9041B	PBGD	26.03	28.1
	PBGD	26.25	
	CK19	ND	33.8
	CK19	ND	
	MGB	29.99	30.4
	MGB	30.5	
745C	PBGD	25.48	26.4
	PBGD	25.37	
	CK19	17.92	19.2
	CK19	17.88	
	MGB	23.93	23.9
	MGB	23.88	
14734B	PBGD	25.79	28.1
	PBGD	25.91	
	CK19	ND	0
	CK19	ND	
	MGB	ND	0
	MGB	ND	

ND = not detected

For two of the samples, the PCR reaction did not detect CK19

For an RNA volume of 1μl, the following Cq values were obtained

Sample	Marker	Cq value	GeneSearch Cq value
9041B	PBGD	26.46	28.1
	PBGD	26.55	
	CK19	ND	33.8
	CK19	ND	
	MGB	30.45	30.4
	MGB	30.83	
745C	PBGD	25.77	26.4
	PBGD	25.8	
	CK19	18.49	19.2
	CK19	18.52	
	MGB	24.43	23.9
	MGB	24.32	
14734B	PBGD	26.26	28.1
	PBGD	26.54	
	CK19	ND	0
	CK19	ND	
	MGB	ND	0
	MGB	ND	

For an RNA volume of 5µl, the following Cq values were obtained

Sample	Marker	Cq value	GeneSearch Cq value
9041B	PBGD	28.83	28.1
	PBGD	28.53	
	CK19	ND	33.8
	CK19	ND	
	MGB	31.91	30.4
	MGB	31.73	
745C	PBGD	26.17	26.4
	PBGD	27.83	
	CK19	17.75	19.2
	CK19	20.77	
	MGB	23.16	23.9
	MGB	24.46	
14734B	PBGD	26.5	28.1
	PBGD	26.21	
	CK19	ND	0
	CK19	ND	
	MGB	ND	0
	MGB	ND	

2.2   The table compares the Cq values of positive nodes run on GeneSearch and the LC480

Patient	Node Number	Histology	Node	PBGD-G	CK19-G	MGB-G	PBGD-480	CK19-480	MGB-480
	H08-								
1	12439	positive	B SN	33.4	19.7	17.2	26.73	19.31	17.8
		positive	C SN	30.3	21.1	18.4	27.16	21.29	18.48
2	13265	positive	B SN	29.3	18.3	20.9	25.79	17.68	21.16
3	15461	positive	B SN	29.1	23.8	25.7	26.86	26.86	26.1
4	16836	positive	B SN	25.2	19.5	0	25.37	19.35	35.83
5	16837	positive	B SN	27.3	20.5	0	26.02	23.6	0
6	16922	positive	B SN	28.9	18.4	18.3	25.58	17.88	17.67
		positive	C SN	32.3	19.2	18.9	25.91	16.89	17.44
		positive	D SN	34.5	16.8	17.1	25.37	18.3	16.91
7	17635	positive	B2 SN	27.2	29.9	31.7	25.78	27.7	33.45
	H09-								
8	7867	positive	D SN	27.3	27.6	24.7	26.09	26.78	26.27
9	8759	positive	B SN	26.4	25.2	0	25.47	23.79	37.14
		positive	C SN	23.8	19.4	35.1	23.92	19.45	33.9
		positive	D SN	23.9	16.1	26.3	23.19	15.61	26.49
		positive	E SN	25.2	19	33.9	24.94	19.56	33.9
		positive	F SN	26.6	20.2	0	24.93	19.98	33.81
		positive	G SN	29.1	21.2	35.4	26.4	20.47	34.77
		positive	H SN	26.4	19.3	34.7	24.75	18.47	34.98
		positive	I SN	26.4	20.5	26.5	25.32	20	27.05
10	9041	negative	B SN	28.1	33.8	30.4	25.86	0	31.56
11	10762	positive	D SN	32.8	19.8	16.1	25.92	19.6	16.84
		positive	E SN	33.9	18.4	15.6	26.19	18.11	16.06
12	10931	positive	B SN	28.4	31.8	27.1	26.79	0	27.87
13	12395	positive	B SN	28.6	0	0	27.12	0	0
14	13447	positive	D SN	32.6	19.5	18.3	26.21	19.65	18.51
		positive	E SN	33.3	19.3	18.2	26.63	18.97	17.6
15	15131	positive	B SN	25.7	31.6	30.8	26.11	0	32.78
16	16039	positive	B SN	25.7	21.6	21.4	25.94	20.43	20.63

		positive	C SN	25.7	20.2	18.6	25.53	19.71	18.46
17	17502	positive	B SN	25.9	19.2	22.1	24.84	17.88	22.58
		positive	C SN	26.1	19.8	24.4	26.23	19.98	24.86
18	18243	positive	B SN	27.6	22.5	18.1	26.69	22.69	18.8
		positive	C SN	27	0	29.5	26.62	0	30.03
	H10-								
19	745	positive	B SN	24.8	22.4	29.2	26.59	23.46	30.94
		positive	C SN	26.4	19.2	23.9	26.67	19.17	25.03
20	925	positive	B SN	27.5	22.8	34.3	27.24	22.8	35.32

2.3 The table compares the Cq values of the negative nodes run on GeneSearch (G) and the LC480

Patient	Node number	Histology	Node	PBGD-G	CK19-G	MGB-G	PBGD-480	CK19-480	MGB-480
	H09								
1	10	negative	B SN	28.6	0	0	26	0	0
		negative	C SN	28.4	0	0	26.66	0	0
2	7867	negative	B SN	27.6	0	0	26.07	0	0
		negative	C SN	27.8	32.8	0	25.98	0	0
		negative	E SN	28.3	34.8	0	26.12	0	0
		negative	F SN	27.8	0	0	26.45	0	0
3	7913	negative	B SN	27.5	0	0	25.99	0	0
		negative	C SN	29.6	0	0	26.71	0	0
4	9041	negative	D SN	28.9	0	0	25.59	0	0
5	9481	negative	B SN	27.9	0	0	26.74	0	0
		negative	C SN	28.8	0	0	26.26	0	0
		negative	D SN	29.4	0	0	26.66	0	0
6	10134	negative	B SN	29.6	0	0	26.94	0	0
		negative	C SN	29.4	0	0	27.05	0	0
		negative	D SN	27.9	0	0	26.17	0	0
		negative	E SN	28.6	0	0	26.29	0	0
7	10136	negative	B SN	27.5	0	0	25.75	0	0
		negative	C SN	27.8	0	0	25.96	0	0
8	10639	negative	B SN	27.8	0	0	25.94	0	0
		negative	C SN	27.4	0	0	25.61	0	0
9	10762	negative	B1 SN	28.6	0	0	26.32	0	0
		negative	B2 SN	28.2	0	0	27.26	0	0
10	10929	negative	B SN	28.7	0	0	26.53	0	0
		negative	C SN	29.2	0	0	26.17	0	0
11	11238	negative	B SN	32	0	32.6	27.47	0	31.36
12	11387	negative	B SN	27.6	0	0	25.87	0	0
		negative	C SN	27.5	33.5	0	26	0	0
		negative	D SN	27.6	0	0	26.71	0	0
13	11531	negative	B2 SN	28.2	0	0	26.18	0	37.04
		negative	D SN	28.5	0	34	26.91	0	33.22
14	11529	negative	B SN	28.6	0	0	27.19	0	0
		negative	C SN	28.6	0	0	26.96	0	37.09
15	12043	negative	B SN	30.2	0	0	27.56	0	0
16	12041	negative	B SN	29.9	0	0	27.56	0	0
17	12393	negative	B SN	28.2	0	0	27.77	0	0
		negative	C SN	27.8	0	0	26.91	0	0
		negative	D SN	29.4	0	0	27.6	0	0

		negative	E SN	27.7	0	0	26.87	0	0
18	12708	negative	B SN	28.8	0	0	26.74	0	0
19	13003	negative	B SN	27.6	0	0	26.43	0	0
		negative	C SN	26.7	0	0	25.81	0	37.86
		negative	D SN	27.3	0	0	26.88	0	0
20	13447	negative	B SN	32.3	0	0	27.49	0	0
21	13643	negative	B1 SN	27.5	0	0	27.18	0	0
		negative	B2 SN	27.5	0	0	26.77	0	0
		negative	C SN	28.5	0	0	27.17	0	0
		negative	D SN	29.6	0	0	27.18	0	35.63
22	13742	negative	B SN	29.9	0	0	27.23	0	0
		negative	C SN	29.5	0	0	27.26	0	0
23	14108	negative	B SN	29.4	0	0	26.68	0	0
		negative	D SN	28.8	0	0	26.68	0	0
		negative	E SN	32	0	0	26.68	0	0
24	14106	negative	B SN	27.5	0	0	26.56	0	37.76
		negative	C SN	26.9	0	0	26.84	0	37.75
25	14732	negative	B SN	29.2	0	34.5	26.66	0	32.21
26	14734	negative	B SN	28.1	0	0	26.69	0	0
		negative	C SN	26.3	0	35.8	25.49	0	34.7
		negative	E SN	26.1	0	0	26.14	0	0
27	15572	negative	B1 SN	27.6	0	0	26.16	0	0
		negative	B2 SN	27.5	0	0	27.31	0	0
28	15570	negative	B2 SN	28.4	0	0	27.32	0	0
		negative	B1 SN	27.8	0	0	26.96	0	0
29	15664	negative	B SN	25.7	0	0	25.57	0	0
		negative	C SN	26.7	0	0	26	0	0
30	15809	negative	B SN	26.3	0	0	25.44	0	0
		negative	C1 SN	26.1	0	0	26.18	0	0
		negative	D SN	26.5	0	0	26.07	0	0
		negative	E SN	26.3	0	0	26.47	0	0
		negative	F SN	27.3	0	0	26.72	0	0
		negative	G SN	26.2	0	0	25.8	0	0
31	15811	negative	B SN	26.3	0	0	25.73	0	0
		negative	C SN	26.7	0	0	26.04	0	0
32	15813	negative	B SN	26.3	0	0	26.68	0	0
		negative	C SN	26.1	0	0	26.72	0	0
33	16202	negative	B SN	26.7	0	0	26.49	0	35.24
34	16204	negative	B SN	27.2	0	0	26.33	0	0
35	16377	negative	B SN	26	0	0	26.45	0	0
36	16757	negative	B SN	27	0	0	26.42	0	0
		negative	C SN	26.6	0	0	26.56	0	0
37	16941	negative	B1 SN	26.2	0	0	25.94	0	0

		negative	B2 SN	26.5	0	0	25.76	0	0
		negative	B3 SN	29.1	0	0	27.04	0	0
38	16945	negative	B SN	26.4	0	0	25.88	0	0
39	16947	negative	B SN	27.2	0	0	26.11	0	0
		negative	C SN	26.2	0	0	26.1	0	0
40	17075	negative	B SN	27.3	0	0	26.26	0	0
41	17151	negative	B SN	25.6	0	0	25.87	0	0
42	17498	negative	B1 SN	26.1	0	0	25.44	0	0
		negative	B2 SN	27.2	0	0	27.16	0	0
43	17500	negative	B SN	26.5	0	0	26.35	0	40
		negative	C SN	27.7	0	0	26.83	0	0
44	18014	negative	B SN	28.6	0	0	26.98	0	0
		negative	C SN	26.9	0	0	26.66	0	0
45	18016	negative	B SN	26.5	0	0	26.68	0	0
46	18155	negative	B SN	27.1	0	0	25.93	0	0
		negative	C1 SN	26.9	0	0	26.49	0	0
47	18157	negative	B1 SN	26.2	0	0	26.74	0	0
		negative	B2 SN	27.8	0	0	27.17	0	0
48	18241	negative	B SN	27.9	0	0	27.12	0	0
		negative	C SN	28.2	0	0	27.13	0	0
49	18472	negative	B1 SN	26.8	0	0	25.93	0	0
		negative	C SN	27.8	0	0	26.52	0	0
50	18525	negative	B SN	26.2	0	0	26.68	0	0
51	18609	negative	B SN	29	0	0	27.44	0	0
		negative	C SN	25.6	33.5	0	25.61	0	0
		negative	D SN	25	0	0	27.56	0	0
	H-10								
52	577	negative	B SN	26.6	0	0	26.54	0	0

2.4 **The table shows the results of all positive patients, comparing GeneSearch (GS) and Metasin Cq values**

(Red highlights discordant results)

Patient	Specimen	GS PBGD Cq Value	Metasin PBGD Cq Value	GS CK19 Cq Value	Metasin CK19 Cq Value	GS MGB Cq Value	Metasin MGB Cq Value
1	08-12439B	33.4	26.9	19.7	18.1	17.2	16.5
	08-12439C	30.3	26.4	21.1	19.2	18.4	17
2	08-13265B	29.3	29.2	18.3	19.3	20.9	23.5
3	08-15461B	29.1	28.6	23.8	24.4	25.7	27.9
4	08-16922D	34.5	26.5	16.8	16.3	17.1	20.7
5	H09-10762D	32.8	26.5	19.8	18.1	16.1	15.3
	H09-10762E	33.9	26.1	18.4	15.5	16.1	15.5
6	H09-10931B	28.4	26.2	31.8	31.7	27.1	25.6
7	H09-11531C1	31.4	24.9	18.9	15.7	18	17.5
	H09-11531C2	32.9	25.3	17.5	16.5	16.6	15.9
	H09-11531D	28.5	28.2	0	0	34	37.9
8	H09-13447B	32.3	27.8	0	0	0	0
	H09-13447C	32.9	28.2	0	0	35.4	33
	H09-13447D	32.6	25.4	19.5	16.8	18.3	16.5
	H09-13447E	33.3	24.7	19.3	16.6	18.2	16.7
9	H09-15131B	25.7	25.1	31.6	0	30.8	31.7
	H09-15131C	26.3	24.8	0	0	35.8	35.1
	H09-15131D	33.4	25.4	0	0	0	0
	H09-15131E	26.1	25.3	0	0	0	0
10	H09-16039B	25.7	24.8	21.6	17.8	21.4	19.8
	H09-16039C	25.7	26.7	20.2	16.4	18.6	16.2
11	H09-17502B	25.9	24.2	19.2	15.6	22.1	21.6
	H09-17502C	26.1	24.2	19.8	16.2	24.4	23
12	H09-18243B	27.6	27.4	22.5	22.3	18.1	20.6
	H09-18243C	27	24.9	0	20.9	29.5	30.4
13	H09-7867B	27.6	25.6	0	0	0	41.7
	H09-7867C	27.8	25.8	32.8	0	0	0
	H09-7867D	27.3	25.7	27.6	27.7	24.7	23.7
	H09-7867E	28.3	25.6	34.8	0	0	40.9
14	H09-8759B	26.4	24.5	25.2	23.3	0	0
	H09-8759C	23.8	23.9	19.4	19.1	35.1	0

	H09-8759D	23.9	24.5	16.1	17	26.3	30.5
	H09-8759E	25.2	25.1	19	18.9	33.9	33.4
	H09-8759F	26.6	24	20.2	18.6	0	32.5
	H09-8759G	29.1	24.5	21.2	17.7	35.4	31.4
	H09-8759H	26.4	24.1	19.3	17.1	34.7	30.9
	H09-8759I	26.4	25.1	20.5	19	26.5	24.1
15	H09-9041B	28.1	25.4	33.8	0	30.4	29.8
	H09-9041C	27.4	28.4	35	0	0	0
	H09-9041D	28.9	29.6	0	0	0	0
16	H10-2344B	26.5	25.6	0	0	0	0
	H10-2344C	27.5	25.9	30.4	0	29.4	27.8
	H10-2344D	30	34.4	0	0	0	0
17	H10-3256B	26.4	26.3	0	0	0	0
	H10-3256C	25	24.8	0	0	0	0
	H10-3256D	25.4	24.4	0	0	0	0
	H10-3256E	26.5	25.7	26.8	28.4	30.5	29.9
18	H10-3258A1	24.1	24.6	19.3	27.4	28.5	0
	H10-3258A2	24.8	24.3	20.4	19.1	30.3	30.4
	H10-3258B	26.9	26.7	27.9	0	0	34.4
	H10-3258C1	27.7	25.8	29.5	0	0	0
	H10-3258C2	27.8	25.4	28.7	0	0	28.4
19	H10-4108A1	28.2	26.2	20.5	18.7	17.3	15.5
	H10-4108A2	26.2	26.2	24.1	23	28	28.5
	H10-4108A3	26.5	25.8	0	0	0	0
20	H10-5068B	31	25.2	25	20.2	26.3	23.4
	H10-5068C	25.5	24.9	23.1	21.8	29.9	30.8
21	H10-5070B	25.9	23.8	18.5	16.5	19.3	17.9
	H10-5070C	27.2	25.6	19	17.8	18.9	18.1
22	H10-5799E1	29.5	25.7	19.2	18.3	14.4	13.8
	H10-5799E2	28.8	28.3	0	0	26.2	26.2
	H10-5799F	30.6	25.6	16.8	16.2	13.2	12.6
23	H10-6173B	24.9	25.3	21.2	20.4	22.2	22.9
24	H10-6399B1	25.5	26.4	30.2	0	34.7	0
	H10-6399B2	27	26.8	24.1	23.3	27	27.2
25	H10-6487B	26.1	26.3	19.4	19.6	19.7	21.3
	H10-6487C	25.3	25.9	26.6	28.3	25.3	26.2
26	H10-6548B	24.3	24.5	19.7	17.2	26.7	28.5
	H10-6548C	26.4	28	0	0	0	0
	H10-6548D	25.9	25.9	23.2	21.2	31.8	34.2
	H10-6548E	28.4	28.5	0	0	0	0
27	H10-6949A1	29.4	29.3	21.7	19.5	22.4	22.2
	H10-6949A2	30.4	27.4	22.1	18.2	22.4	19.8
	H10-6949C	26.2	26.2	0	0	0	0

28	H10-745B	24.8	24.3	22.4	16.3	29.2	22.3
	H10-745C	26.4	24.3	19.2	16.1	23.9	28.3
29	H10-7525B	26.2	25.6	19	16.2	0	0
	H10-7525C	25.9	27.5	0	0	0	0
	H10-7525D	29.8	27.9	22.7	19.9	0	0
	H10-7525E	27	28.4	0	0	0	0
30	H10-7587B	28.6	30.5	30.4	0	31.5	34.9
	H10-7587C	27.9	27.3	26.5	25.3	28.3	29.5
	H10-7587D1	31.7	31	0	0	0	0
31	H10-7638B	25	22.9	20	16.6	20.1	19.2
32	H10-8489B	25.5	26.3	29.8	31.3	29.8	32.3
	H10-8489C	27	26.5	0	0	0	0
33	H10-9031B	26.5	27.2	21.8	19.3	27.6	28.3
	H10-9031C	27.2	27.7	0	0	0	0
	H10-9031D	27.4	28.3	0	0	0	0
34	H10-9153B	29.9	30	24.9	25.5	22.4	23.2
	H10-9153C	27.1	28.8	0	0	35.3	38.6
	H10-9153F	27.5	29.6	0	0	0	0
35	H10-925B	27.5	27.1	22.8	20.5	34.3	34.4
36	H10-9351B1	27.8	30.9	21.4	21.9	22.5	24.1
	H10-9351B2	28.7	29.8	20.2	20.3	20.2	20.5
37	H10-9442B	27.5	29.2	28.3	0	27.1	29.2
	H10-9442C1	26.6	27.2	0	0	0	0
	H10-9442C2	28.8	29.6	0	0	0	0

2.5 The table below compares the Cq values of all the positive <u>nodes</u> of GeneSearch (GS) and Metasin

(Red highlights discordant results)

Node	Specimen	GS PBGD Cq Value	Metasin PBGD Cq Value	GS CK19 Cq Value	Metasin CK19 Cq Value	GS MGB Cq Value	Metasin MGB Cq Value
1	08-12439B	33.4	26.9	19.7	18.1	17.2	16.5
2	08-12439C	30.3	26.4	21.1	19.2	18.4	17
3	08-13265B	29.3	29.2	18.3	19.3	20.9	23.5
4	08-15461B	29.1	28.6	23.8	24.4	25.7	27.9
5	08-16922D	34.5	26.5	16.8	16.3	17.1	20.7
6	H09-10762D	32.8	26.5	19.8	18.1	16.1	15.3
7	H09-10762E	33.9	26.1	18.4	15.5	16.1	15.5
8	H09-10931B	28.4	26.2	31.8	31.7	27.1	25.6
9	H09-11531C1	31.4	24.9	18.9	15.7	18	17.5
10	H09-11531C2	32.9	25.3	17.5	16.5	16.6	15.9
11	H09-13447D	32.6	25.4	19.5	16.8	18.3	16.5
12	H09-13447E	33.3	24.7	19.3	16.6	18.2	16.7
13	H09-15131B	25.7	25.1	31.6	0	30.8	31.7
14	H09-16039B	25.7	24.8	21.6	17.8	21.4	19.8
15	H09-16039C	25.7	26.7	20.2	16.4	18.6	16.2
16	H09-17502B	25.9	24.2	19.2	15.6	22.1	21.6
17	H09-17502C	26.1	24.2	19.8	16.2	24.4	23
18	H09-18243B	27.6	27.4	22.5	22.3	18.1	20.6
19	H09-18243C	27	24.9	0	20.9	29.5	30.4
20	H09-7867D	27.3	25.7	27.6	27.7	24.7	23.7
21	H09-8759B	26.4	24.5	25.2	23.3	0	0
22	H09-8759C	23.8	23.9	19.4	19.1	35.1	0
23	H09-8759D	23.9	24.5	16.1	17	26.3	30.5
24	H09-8759E	25.2	25.1	19	18.9	33.9	33.4
25	H09-8759F	26.6	24	20.2	18.6	0	32.5
26	H09-8759G	29.1	24.5	21.2	17.7	35.4	31.4
27	H09-8759H	26.4	24.1	19.3	17.1	34.7	30.9
28	H09-8759I	26.4	25.1	20.5	19	26.5	24.1
29	H09-9041B	28.1	25.4	33.8	0	30.4	29.8
30	H10-2344C	27.5	25.9	30.4	0	29.4	27.8
31	H10-3256E	26.5	25.7	26.8	28.4	30.5	29.9
32	H10-3258A1	24.1	24.6	19.3	27.4	28.5	0
33	H10-3258A2	24.8	24.3	20.4	19.1	30.3	30.4

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34	H10-3258C2	27.8	25.4	28.7	0	0	28.4
35	H10-4108A1	28.2	26.2	20.5	18.7	17.3	15.5
36	H10-4108A2	26.2	26.2	24.1	23	28	28.5
37	H10-5068B	31	25.2	25	20.2	26.3	23.4
38	H10-5068C	25.5	24.9	23.1	21.8	29.9	30.8
39	H10-5070B	25.9	23.8	18.5	16.5	19.3	17.9
40	H10-5070C	27.2	25.6	19	17.8	18.9	18.1
41	H10-5799E1	29.5	25.7	19.2	18.3	14.4	13.8
42	H10-5799E2	28.8	28.3	0	0	26.2	26.2
43	H10-5799F	30.6	25.6	16.8	16.2	13.2	12.6
44	H10-6173B	24.9	25.3	21.2	20.4	22.2	22.9
45	H10-6399B2	27	26.8	24.1	23.3	27	27.2
46	H10-6487B	26.1	26.3	19.4	19.6	19.7	21.3
47	H10-6487C	25.3	25.9	26.6	28.3	25.3	26.2
48	H10-6548B	24.3	24.5	19.7	17.2	26.7	28.5
49	H10-6548D	25.9	25.9	23.2	21.2	31.8	34.2
50	H10-6949A1	29.4	29.3	21.7	19.5	22.4	22.2
51	H10-6949A2	30.4	27.4	22.1	18.2	22.4	19.8
52	H10-745B	24.8	24.3	22.4	16.3	29.2	22.3
53	H10-745C	26.4	24.3	19.2	16.1	23.9	28.3
54	H10-7525B	26.2	25.6	19	16.2	0	0
55	H10-7525D	29.8	27.9	22.7	19.9	0	0
56	H10-7587C	27.9	27.3	26.5	25.3	28.3	29.5
57	H10-7638B	25	22.9	20	16.6	20.1	19.2
58	H10-8489B	25.5	26.3	29.8	31.3	29.8	32.3
59	H10-9031B	26.5	27.2	21.8	19.3	27.6	28.3
60	H10-9153B	29.9	30	24.9	25.5	22.4	23.2
61	H10-925B	27.5	27.1	22.8	20.5	34.3	34.4
62	H10-9351B1	27.8	30.9	21.4	21.9	22.5	24.1
63	H10-9351B2	28.7	29.8	20.2	20.3	20.2	20.5
64	H10-9442B	27.5	29.2	28.3	0	27.1	29.2

2.6 Table of all patients found negative on GeneSearch (GS) and Metasin

Patient	Specimen	GS PBGD Cq Value	Metasin PBGD Cq Value	GS CK19 Cq Value	Metasin CK19 Cq Value	GS MGB Cq Value	Metasin MGB Cq Value
1	08-10986B	28.2	26.3	31.3	0	0	0
2	08-11058A	29.9	26.9	0	0	0	0
	08-11058B	28	27	0	0	0	0
3	08-11465A	28.1	26.8	0	0	0	0
	08-11465B	27.1	26.7	0	0	0	0
	08-11465C	27.4	25.6	0	0	0	0
	08-11465D	27.3	25.6	0	0	0	0
	08-11465E	27.8	26.5	0	0	0	0
4	08-11753A	28.6	26.7	0	0	0	0
	08-11753B	29.2	28	0	0	0	0
	08-11753C	27.5	26.8	0	0	0	0
5	08-11839B1	27.9	27.2	0	0	0	0
	08-11839B2	27.7	26.4	0	0	0	0
	08-11839B3	27.6	26.4	0	0	0	0
	08-11839C1	28.8	26.4	0	0	0	0
	08-11839C2	27.6	26.6	0	0	0	0
	08-11839C3	31.4	28.6	0	0	0	0
6	08-12488B1	27.6	27	0	0	0	0
	08-12488B2	27.4	25.9	0	0	0	0
7	08-12779B	27.3	26.1	0	0	0	0
	08-12779C	28.3	26.4	0	0	0	0
8	08-12781B	27.9	25.6	0	0	0	0
9	08-14328B	28.2	26.7	0	0	0	0
	08-14328C	26	25.7	0	0	0	0
10	08-14714B	27.8	25.9	0	0	0	0
	08-14714C	27.6	25.7	0	0	0	0
11	08-14920B	29.7	26.8	0	0	0	0
12	08-15390B	28.1	25.2	0	0	0	0
	08-15390C	27.6	25.2	0	0	0	0
13	08-15833B	27.4	26.1	0	0	0	0
14	08-17257B	27.7	26.1	0	0	0	0
15	08-17325B	29.7	25.3	0	33.7	0	0
	08-17325C	28.4	25.5	0	0	0	0
	08-17325D	27.8	24.7	0	33.3	0	0

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	08-17325E	28.1	25.4	0	34.4	0	0
	08-17325F	27.8	25.4	0	33.9	0	0
16	08-17631B	29.2	26	0	0	0	0
	08-17631C	28.2	25.9	0	0	0	0
17	08-17633B	27.5	25.5	0	0	0	0
	08-17633C	27	24.1	34	0	0	0
18	08-17779B1	29.6	25.4	35.8	0	0	44.3
	08-17779B2	30.8	24.7	0	0	0	0
19	08-17781B	31.8	28.5	0	0	0	0
20	08-18324B	28.1	25.6	0	0	0	0
21	08-18475B	29.7	25.7	0	0	0	0
22	08-18477B	29.1	25.8	0	0	0	0
	08-18477C	29.3	25.9	0	0	0	0
23	08-18857B	28.2	25.1	34.9	0	0	0
24	H09-10134B	29.6	26.9	0	0	0	0
	H09-10134C	29.4	26.3	0	0	0	0
	H09-10134D	27.9	25.2	0	0	0	0
	H09-10134E	28.6	24.5	0	0	0	0
25	H09-10136B	27.5	24.6	0	0	0	0
	H09-10136C	27.8	24.7	0	0	0	0
26	H09-10639B	27.8	25.6	0	0	0	0
	H09-10639C	27.4	24.4	0	0	0	37.5
27	H09-10929B	28.7	27	0	0	0	0
	H09-10929C	29.2	25.7	0	0	0	0
28	H09-11387B	27.6	25.5	0	0	0	39.9
	H09-11387C	27.5	25.4	33.5	0	0	0
	H09-11387D	27.6	25.8	0	0	0	43.8
29	H09-11529B	28.6	26	0	0	0	0
	H09-11529C	28.6	26.1	0	0	0	0
30	H09-12041B	30.2	27.4	0	0	0	0
31	H09-12043B	29.9	27.4	0	0	0	0
32	H09-12393B	28.2	24.6	0	0	0	0
	H09-12393C	27.8	25.2	0	0	0	0
	H09-12393D	29.4	27.1	0	0	0	40.6
	H09-12393E	27.7	26.2	0	0	0	42.6
33	H09-12395B	28.6	26.1	0	0	0	0
34	H09-12708B	28.8	25.7	0	0	0	0
35	H09-13003B	27.6	24.9	0	0	0	0
	H09-13003C	26.7	24.4	0	0	0	0
	H09-13003D	27.3	25.5	0	0	0	0
36	H09-13643B1	27.5	24.6	0	0	0	0
	H09-	28.5	25.2	0	0	0	0

	13643B2						
	H09-13643C	29.6	27	0	0	0	0
	H09-13643D	27.7	25.2	0	0	0	35.7
37	H09-13742B	29.9	25.7	0	0	0	0
	H09-13742C	29.5	26.3	0	0	0	0
38	H09-14108C	29.4	27.9	0	0	0	0
	H09-14108D	30.7	29	0	0	0	0
	H09-14108E	28.8	27.3	0	0	0	0
	H09-14108F	32	30.3	0	0	0	0
39	H09-14590B	28.5	26.1	0	0	0	0
	H09-14590C	29.7	26.2	0	0	0	0
	H09-14590D	27.3	25.9	0	0	0	0
	H09-14590E	27.7	26.4	0	0	0	0
	H09-14590F	26.6	26.4	0	0	0	0
40	H09-14592B	27.4	26.8	0	0	0	0
41	H09-14732B	29.2	26.3	0	0	34.5	32.7
42	H09-14734B	28.1	25.8	0	0	0	0
43	H09-15535B	26.4	25.1	0	0	0	0
	H09-15535C	26.5	25.9	0	0	0	0
	H09-15535D	28.4	26.6	0	0	0	0
44	H09-15570B	28.4	28.3	0	0	0	0
	H09-15570C	27.8	26.3	0	0	0	0
45	H09-15572B1	27.6	25.7	0	0	0	0
	H09-15572B2	27.5	28.7	0	0	0	0
46	H09-15664B	25.7	26.1	0	0	0	0
	H09-15664C	26.7	26	0	0	0	0
47	H09-15809B	26.3	25.2	0	0	0	0
	H09-15809C	26.1	25.7	0	0	0	0
	H09-15809D	26.6	26.3	0	0	0	0
	H09-15809E	25.8	26.1	0	0	0	0
	H09-15809F	26.5	26.3	0	0	0	0
	H09-15809G	26.3	25.4	0	0	0	0
48	H09-15811B	26.3	26.2	0	0	0	0
	H09-15811C	26.7	26.1	0	0	0	0
49	H09-16202B	26.7	25.4	0	0	0	35
50	H09-16204B	27.2	25.7	0	0	0	0
51	H09-16377B	26	24.8	0	0	0	0
52	H09-16757B	27	25.9	0	0	0	0
	H09-16757C	26.6	25.6	0	0	0	0
53	H09-16941B1	26.2	25.4	0	0	0	0

	H09-16941B2	26.5	24.8	0	0	0	34.4
	H09-16941B3	29.1	25.7	0	0	0	0
54	H09-16945B	26.4	25.4	0	0	0	0
55	H09-16947B	27.2	25.2	0	0	0	0
	H09-16947C	26.2	25.5	0	0	0	0
56	H09-17075B	27.3	25.8	0	0	0	0
57	H09-17151B	25.6	25.5	0	0	0	0
58	H09-17498B1	26.1	24.8	0	0	0	0
	H09-17498B2	27.2	25.4	0	0	0	0
59	H09-17500B	26.5	25	0	0	0	0
	H09-17500C	27.7	25.1	0	0	0	0
60	H09-18014B	28.6	27	0	0	0	0
	H09-18014C	26.9	27	0	0	0	0
61	H09-18016B	26.5	24.9	0	0	0	0
62	H09-18155B	27.1	26	0	0	0	0
	H09-18155C1	26.9	27	0	0	0	0
	H09-18155C2	26.5	25.4	0	0	0	0
63	H09-18157B	26.2	26.1	0	0	0	0
	H09-18157C	27.8	26.3	0	0	0	0
64	H09-18241B	27.9	26.7	0	0	0	0
	H09-18241C	28.2	26.5	0	0	0	0
65	H09-18472B1	26.8	25.8	0	0	0	0
	H09-18472B2	32.1	30.6	0	0	0	0
	H09-18472C	27.8	26.2	0	0	0	0
66	H09-18525B	26.2	25.9	0	0	0	0
67	H09-18609B	29	26.4	0	0	0	0
	H09-18609C	25.6	24.7	33.5	0	0	0
	H09-18609D	25	25.6	0	0	0	43.7
68	H09-7913B	27.5	25.4	0	0	0	0
	H09-7913C	29.6	27.3	0	0	0	0
69	H09-7915B1	30.5	27.3	0	0	0	0
	H09-7915C	29.1	30.2	0	0	0	0
70	H09-7965B1	28.7	29.1	0	0	0	0
	H09-7965B2	28.5	28.8	0	0	0	0
71	H09-8667C	29.5	29.7	0	0	0	0

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72	H09-9039B	28.9	29.7	0	0	0	0
	H09-9039C	28.5	26.8	0	0	0	42.4
73	H09-9145B	28.4	29.3	0	0	0	0
	H09-9145C	28.5	29.7	0	0	0	0
	H09-9145D	28.1	28.8	0	0	0	0
	H09-9145E1	30.1	29.7	0	0	0	0
	H09-9145E2	30.2	28.2	0	0	0	0
74	H09-9481B	27.9	28.3	0	0	0	0
	H09-9481C	28.8	26.4	0	0	0	0
	H09-9481D	29.4	25.8	0	0	0	0
75	H10-1294B	28.2	27	0	0	0	0
	H10-1294C	27.5	26.3	0	0	0	0
76	H10-1443B1	26.1	26	32.9	0	0	0
	H10-1443B2	26.4	25.1	36	0	0	0
	H10-1443D	26.5	25.4	0	0	0	0
77	H10-1445B	25.5	24	33.9	0	0	0
	H10-1445C	25.8	24.2	33.1	0	0	0
	H10-1445D	26.3	25.2	34	0	0	0
	H10-1445E	25.9	25.1	35	0	0	0
78	H10-1978B	28	27	0	0	0	0
	H10-1978C	26.6	25.7	0	0	0	0
	H10-1978D	26.5	24.6	0	0	0	0
79	H10-2487B	25.3	24.8	0	0	0	0
	H10-2487C	26.1	24.8	0	0	0	0
80	H10-2618A	25.9	27.2	0	0	0	43.1
	H10-2618B	25.4	28.3	0	0	0	0
81	H10-2620A	26.3	25.3	0	0	0	0
82	H10-2712B	26.2	32	0	0	0	0
	H10-2712C	25.3	27.1	33.9	0	0	0
	H10-2712D	26.6	33	0	0	0	0
83	H10-2920B	28.2	26.8	0	0	0	0
	H10-2920C	28.3	26.4	0	0	0	0
	H10-2920D	26.4	25.9	0	0	0	0
84	H10-3302B1	26.7	27	0	0	0	0
	H10-3302B5	24.9	25.6	32.5	0	0	0
85	H10-3466B	27.3	26.8	0	0	0	0
	H10-3466C	27.2	25.9	0	0	0	0
86	H10-3635B	25.3	25.2	0	0	0	0
	H10-3635C	25.7	25.8	0	0	0	0
	H10-3635D	25.8	25	0	0	0	0
87	H10-3820B	26.3	26	0	0	0	0
88	H10-3989B	24.7	24.5	0	0	0	0
	H10-3989C	26.2	25.8	0	0	0	0

	H10-3989D	25.8	24.9	0	0	0	0
89	H10-3991A	27.2	29.8	0	0	0	0
	H10-3991B	25.9	26	0	0	0	0
90	H10-4499B	25.3	30.7	0	0	0	0
	H10-4499C	28.9	28.4	0	0	0	0
	H10-4499D	27.3	31.5	0	0	0	0
	H10-4499E	26.5	25.4	0	0	0	0
91	H10-4945B	29.3	25.4	0	0	0	0
	H10-4945C	27.2	28.1	0	0	0	0
	H10-4945D	27.3	26.3	0	0	0	0
92	H10-5123B	25.1	27.1	0	0	0	0
	H10-5123C	25.1	27.7	0	0	0	0
	H10-5123D	27.1	28.6	0	0	0	0
93	H10-5675B	25.9	27.6	0	0	0	0
	H10-5675C	27.5	28.8	0	0	0	0
94	H10-577B	26.6	24.8	0	0	0	0
95	H10-5809B	26.2	26.4	0	0	0	0
	H10-5809C	27.2	27	0	0	0	0
96	H10-6546B	26.8	25.3	0	0	0	33
	H10-6546C	26.3	28.2	0	0	0	0
	H10-6546D	28.1	28.7	0	0	0	0
97	H10-6949C	26.2	26.2	0	0	0	0
98	H10-7287B	26.1	27.3	0	0	0	0
99	H10-7375B	26.1	27.5	0	0	0	0
100	H10-747B	26.1	25.1	0	0	0	0
	H10-747C	27.4	25	0	0	0	0
101	H10-8267A	27.4	28.7	0	0	0	0
	H10-8267B1	28.7	29.5	0	0	0	0
	H10-8267B2	28.1	28.3	0	0	0	0
102	H10-8352B	26.9	27.7	0	0	0	0
	H10-8352C	27.5	27.2	0	0	0	0
103	H10-8528B	26.1	28.2	34.9	0	0	0
104	H10-8737B	26.7	26.2	0	0	0	0
	H10-8737C	27	26.4	0	0	0	0
105	H10-8936B	25.3	29.9	0	0	0	0
	H10-8936C	25.5	28.7	0	0	0	0
	H10-8936D	26.6	29.7	0	0	0	0
106	H10-8938B	27.3	30	0	0	0	0
	H10-8938C	25.2	26.8	0	0	0	0
107	H10-9033B	26.6	27.9	0	0	0	0
	H10-9033C	26.2	28	0	0	0	0
	H10-9033D	26.7	29.1	0	0	0	0
108	H10-9308B	26.6	29.2	0	0	0	0

	H10-9308C	25.8	27.1	0	0	0	0
	H10-9308D	26.3	27.8	0	0	0	0
109	H10-9310B	27	27.6	0	0	0	38.8
	H10-9310C	28.1	29	0	0	0	0
	H10-9310D	27.5	28.9	0	0	0	0
110	H10-9442C1	26.6	27.2	0	0	0	0
	H10-9442C2	28.8	29.6	0	0	0	0
111	H10-9444B	25.7	26.4	0	0	0	0

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2.7   Table of all nodes found negative on GeneSearch (GS) and Metasin

Patient	Specimen	GS PBGD Cq Value	Metasin PBGD Cq Value	GS CK19 Cq Value	Metasin CK19 Cq Value	GS MGB Cq Value	Metasin MGB Cq Value
1	08-10986B	28.2	26.3	31.3	0	0	0
2	08-11058A	29.9	26.9	0	0	0	0
	08-11058B	28	27	0	0	0	0
3	08-11465A	28.1	26.8	0	0	0	0
	08-11465B	27.1	26.7	0	0	0	0
	08-11465C	27.4	25.6	0	0	0	0
	08-11465D	27.3	25.6	0	0	0	0
	08-11465E	27.8	26.5	0	0	0	0
4	08-11753A	28.6	26.7	0	0	0	0
	08-11753B	29.2	28	0	0	0	0
	08-11753C	27.5	26.8	0	0	0	0
5	08-11839B1	27.9	27.2	0	0	0	0
	08-11839B2	27.7	26.4	0	0	0	0
	08-11839B3	27.6	26.4	0	0	0	0
	08-11839C1	28.8	26.4	0	0	0	0
	08-11839C2	27.6	26.6	0	0	0	0
	08-11839C3	31.4	28.6	0	0	0	0
1	08-12082B1	28.5	26.5	0	0	0	36.2
	08-12082B3	28.5	26.6	0	0	0	36.9
	08-12082C3	29.1	26.4	0	0	35	34.2
6	08-12488B1	27.6	27	0	0	0	0
	08-12488B2	27.4	25.9	0	0	0	0
7	08-12779B	27.3	26.1	0	0	0	0
	08-12779C	28.3	26.4	0	0	0	0
8	08-12781B	27.9	25.6	0	0	0	0
9	08-14328B	28.2	26.7	0	0	0	0
	08-14328C	26	25.7	0	0	0	0
10	08-14714B	27.8	25.9	0	0	0	0
	08-14714C	27.6	25.7	0	0	0	0
11	08-14920B	29.7	26.8	0	0	0	0
12	08-15390B	28.1	25.2	0	0	0	0
	08-15390C	27.6	25.2	0	0	0	0
13	08-15833B	27.4	26.1	0	0	0	0
14	08-17257B	27.7	26.1	0	0	0	0

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15	08-17325B	29.7	25.3	0	33.7	0	0
	08-17325C	28.4	25.5	0	0	0	0
	08-17325D	27.8	24.7	0	33.3	0	0
	08-17325E	28.1	25.4	0	34.4	0	0
	08-17325F	27.8	25.4	0	33.9	0	0
16	08-17631B	29.2	26	0	0	0	0
	08-17631C	28.2	25.9	0	0	0	0
17	08-17633B	27.5	25.5	0	0	0	0
	08-17633C	27	24.1	34	0	0	0
	08-17635B1	27.8	25.6	34.8	0	0	0
18	08-17779B1	29.6	25.4	35.8	0	0	44.3
	08-17779B2	30.8	24.7	0	0	0	0
19	08-17781B	31.8	28.5	0	0	0	0
20	08-18324B	28.1	25.6	0	0	0	0
21	08-18475B	29.7	25.7	0	0	0	0
22	08-18477B	29.1	25.8	0	0	0	0
	08-18477C	29.3	25.9	0	0	0	0
23	08-18857B	28.2	25.1	34.9	0	0	0
	09-15813C	26.1	25.3	0	0	0	0
31	H09-10134B	29.6	26.9	0	0	0	0
	H09-10134C	29.4	26.3	0	0	0	0
	H09-10134D	27.9	25.2	0	0	0	0
	H09-10134E	28.6	24.5	0	0	0	0
32	H09-10136B	27.5	24.6	0	0	0	0
	H09-10136C	27.8	24.7	0	0	0	0
33	H09-10639B	27.8	25.6	0	0	0	0
	H09-10639C	27.4	24.4	0	0	0	37.5
34	H09-10929B	28.7	27	0	0	0	0
	H09-10929C	29.2	25.7	0	0	0	0
35	H09-11387B	27.6	25.5	0	0	0	39.9
	H09-11387C	27.5	25.4	33.5	0	0	0
	H09-11387D	27.6	25.8	0	0	0	43.8
36	H09-11529B	28.6	26	0	0	0	0
	H09-11529C	28.6	26.1	0	0	0	0
	H09-11531D	28.5	28.2	0	0	34	37.9
38	H09-12041B	30.2	27.4	0	0	0	0
37	H09-12043B	29.9	27.4	0	0	0	0
39	H09-12393B	28.2	24.6	0	0	0	0
	H09-12393C	27.8	25.2	0	0	0	0
	H09-12393D	29.4	27.1	0	0	0	40.6
	H09-12393E	27.7	26.2	0	0	0	42.6
40	H09-12395B	28.6	26.1	0	0	0	0
41	H09-12708B	28.8	25.7	0	0	0	0

42	H09-13003B	27.6	24.9	0	0	0	0
	H09-13003C	26.7	24.4	0	0	0	0
	H09-13003D	27.3	25.5	0	0	0	0
	H09-13447B	32.3	27.8	0	0	0	0
	H09-13447C	32.9	28.2	0	0	35.4	33
43	H09-13643B1	27.5	24.6	0	0	0	0
	H09-13643B2	28.5	25.2	0	0	0	0
	H09-13643C	29.6	27	0	0	0	0
	H09-13643D	27.7	25.2	0	0	0	35.7
44	H09-13742B	29.9	25.7	0	0	0	0
	H09-13742C	29.5	26.3	0	0	0	0
45	H09-14108C	29.4	27.9	0	0	0	0
	H09-14108D	30.7	29	0	0	0	0
	H09-14108E	28.8	27.3	0	0	0	0
	H09-14108F	32	30.3	0	0	0	0
46	H09-14590B	28.5	26.1	0	0	0	0
	H09-14590C	29.7	26.2	0	0	0	0
	H09-14590D	27.3	25.9	0	0	0	0
	H09-14590E	27.7	26.4	0	0	0	0
	H09-14590F	26.6	26.4	0	0	0	0
47	H09-14592B	27.4	26.8	0	0	0	0
48	H09-14732B	29.2	26.3	0	0	34.5	32.7
49	H09-14734B	28.1	25.8	0	0	0	0
	H09-15131C	26.3	24.8	0	0	35.8	35.1
	H09-15131D	33.4	25.4	0	0	0	0
	H09-15131E	26.1	25.3	0	0	0	0
50	H09-15535B	26.4	25.1	0	0	0	0
	H09-15535C	26.5	25.9	0	0	0	0
	H09-15535D	28.4	26.6	0	0	0	0
52	H09-15570B	28.4	28.3	0	0	0	0
	H09-15570C	27.8	26.3	0	0	0	0
51	H09-15572B1	27.6	25.7	0	0	0	0
	H09-15572B2	27.5	28.7	0	0	0	0
53	H09-15664B	25.7	26.1	0	0	0	0
	H09-15664C	26.7	26	0	0	0	0
54	H09-15809B	26.3	25.2	0	0	0	0
	H09-15809C	26.1	25.7	0	0	0	0
	H09-15809D	26.6	26.3	0	0	0	0
	H09-15809E	25.8	26.1	0	0	0	0

	H09-15809F	26.5	26.3	0	0	0	0
	H09-15809G	26.3	25.4	0	0	0	0
55	H09-15811B	26.3	26.2	0	0	0	0
	H09-15811C	26.7	26.1	0	0	0	0
56	H09-16202B	26.7	25.4	0	0	0	35
57	H09-16204B	27.2	25.7	0	0	0	0
58	H09-16377B	26	24.8	0	0	0	0
59	H09-16757B	27	25.9	0	0	0	0
	H09-16757C	26.6	25.6	0	0	0	0
60	H09-16941B1	26.2	25.4	0	0	0	0
	H09-16941B2	26.5	24.8	0	0	0	34.4
	H09-16941B3	29.1	25.7	0	0	0	0
61	H09-16945B	26.4	25.4	0	0	0	0
62	H09-16947B	27.2	25.2	0	0	0	0
	H09-16947C	26.2	25.5	0	0	0	0
63	H09-17075B	27.3	25.8	0	0	0	0
64	H09-17151B	25.6	25.5	0	0	0	0
65	H09-17498B1	26.1	24.8	0	0	0	0
	H09-17498B2	27.2	25.4	0	0	0	0
66	H09-17500B	26.5	25	0	0	0	0
	H09-17500C	27.7	25.1	0	0	0	0
67	H09-18014B	28.6	27	0	0	0	0
	H09-18014C	26.9	27	0	0	0	0
68	H09-18016B	26.5	24.9	0	0	0	0
69	H09-18155B	27.1	26	0	0	0	0
	H09-18155C1	26.9	27	0	0	0	0
	H09-18155C2	26.5	25.4	0	0	0	0
70	H09-18157B	26.2	26.1	0	0	0	0
	H09-18157C	27.8	26.3	0	0	0	0
71	H09-18241B	27.9	26.7	0	0	0	0
	H09-18241C	28.2	26.5	0	0	0	0
72	H09-18472B1	26.8	25.8	0	0	0	0
	H09-18472B2	32.1	30.6	0	0	0	0
	H09-18472C	27.8	26.2	0	0	0	0
73	H09-18525B	26.2	25.9	0	0	0	0

74	H09-18609B	29	26.4	0	0	0	0
	H09-18609C	25.6	24.7	33.5	0	0	0
	H09-18609D	25	25.6	0	0	0	43.7
5	H09-7867B	27.6	25.6	0	0	0	41.7
	H09-7867C	27.8	25.8	32.8	0	0	0
	H09-7867E	28.3	25.6	34.8	0	0	40.9
25	H09-7913B	27.5	25.4	0	0	0	0
	H09-7913C	29.6	27.3	0	0	0	0
24	H09-7915B1	30.5	27.3	0	0	0	0
	H09-7915C	29.1	30.2	0	0	0	0
30	H09-7965B1	28.7	29.1	0	0	0	0
	H09-7965B2	28.5	28.8	0	0	0	0
26	H09-8667C	29.5	29.7	0	0	0	0
27	H09-9039B	28.9	29.7	0	0	0	0
	H09-9039C	28.5	26.8	0	0	0	42.4
	H09-9041C	27.4	28.4	35	0	0	0
	H09-9041D	28.9	29.6	0	0	0	0
28	H09-9145B	28.4	29.3	0	0	0	0
	H09-9145C	28.5	29.7	0	0	0	0
	H09-9145D	28.1	28.8	0	0	0	0
	H09-9145E1	30.1	29.7	0	0	0	0
	H09-9145E2	30.2	28.2	0	0	0	0
29	H09-9481B	27.9	28.3	0	0	0	0
	H09-9481C	28.8	26.4	0	0	0	0
	H09-9481D	29.4	25.8	0	0	0	0
77	H10-1294B	28.2	27	0	0	0	0
	H10-1294C	27.5	26.3	0	0	0	0
78	H10-1443B1	26.1	26	32.9	0	0	0
	H10-1443B2	26.4	25.1	36	0	0	0
	H10-1443D	26.5	25.4	0	0	0	0
79	H10-1445B	25.5	24	33.9	0	0	0
	H10-1445C	25.8	24.2	33.1	0	0	0
	H10-1445D	26.3	25.2	34	0	0	0
	H10-1445E	25.9	25.1	35	0	0	0
	10-1643B	28.4	26.7	0	0	0	0
9	10-1643C	27.8	25.6	34.8	0	0	0
80	H10-1978B	28	27	0	0	0	0
	H10-1978C	26.6	25.7	0	0	0	0
	H10-1978D	26.5	24.6	0	0	0	0
18	H10-2344B	26.5	25.6	0	0	0	0
	H10-2344D	30	34.4	0	0	0	0
81	H10-2487B	25.3	24.8	0	0	0	0
	H10-2487C	26.1	24.8	0	0	0	0

82	H10-2618A	25.9	27.2	0	0	0	43.1
	H10-2618B	25.4	28.3	0	0	0	0
83	H10-2620A	26.3	25.3	0	0	0	0
84	H10-2712B	26.2	32	0	0	0	0
	H10-2712C	25.3	27.1	33.9	0	0	0
	H10-2712D	26.6	33	0	0	0	0
85	H10-2920B	28.2	26.8	0	0	0	0
	H10-2920C	28.3	26.4	0	0	0	0
	H10-2920D	26.4	25.9	0	0	0	0
19	H10-3256B	26.4	26.3	0	0	0	0
	H10-3256C	25	24.8	0	0	0	0
	H10-3256D	25.4	24.4	0	0	0	0
86	H10-3302B1	26.7	27	0	0	0	0
	H10-3302B5	24.9	25.6	32.5	0	0	0
87	H10-3466B	27.3	26.8	0	0	0	0
	H10-3466C	27.2	25.9	0	0	0	0
88	H10-3635B	25.3	25.2	0	0	0	0
	H10-3635C	25.7	25.8	0	0	0	0
	H10-3635D	25.8	25	0	0	0	0
89	H10-3820B	26.3	26	0	0	0	0
90	H10-3989B	24.7	24.5	0	0	0	0
	H10-3989C	26.2	25.8	0	0	0	0
	H10-3989D	25.8	24.9	0	0	0	0
91	H10-3991A	27.2	29.8	0	0	0	0
	H10-3991B	25.9	26	0	0	0	0
	H10-4108A3	26.5	25.8	0	0	0	0
92	H10-4499B	25.3	30.7	0	0	0	0
	H10-4499C	28.9	28.4	0	0	0	0
	H10-4499D	27.3	31.5	0	0	0	0
	H10-4499E	26.5	25.4	0	0	0	0
93	H10-4945B	29.3	25.4	0	0	0	0
	H10-4945C	27.2	28.1	0	0	0	0
	H10-4945D	27.3	26.3	0	0	0	0
94	H10-5123B	25.1	27.1	0	0	0	0
	H10-5123C	25.1	27.7	0	0	0	0
	H10-5123D	27.1	28.6	0	0	0	0
95	H10-5675B	25.9	27.6	0	0	0	0
	H10-5675C	27.5	28.8	0	0	0	0
75	H10-577B	26.6	24.8	0	0	0	0
96	H10-5809B	26.2	26.4	0	0	0	0
	H10-5809C	27.2	27	0	0	0	0
28	H10-6399B1	25.5	26.4	30.2	0	34.7	0
97	H10-6546B	26.8	25.3	0	0	0	33

	H10-6546C	26.3	28.2	0	0	0	0
	H10-6546D	28.1	28.7	0	0	0	0
	H10-6548C	26.4	28	0	0	0	0
	H10-6548E	28.4	28.5	0	0	0	0
98	H10-6949C	26.2	26.2	0	0	0	0
99	H10-7287B	26.1	27.3	0	0	0	0
100	H10-7375B	26.1	27.5	0	0	0	0
76	H10-747B	26.1	25.1	0	0	0	0
	H10-747C	27.4	25	0	0	0	0
	H10-7525C	25.9	27.5	0	0	0	0
	H10-7525E	27	28.4	0	0	0	0
33	H10-7587B	28.6	30.5	30.4	0	31.5	34.9
	H10-7587D1	31.7	31	0	0	0	0
	H10-8267A	28.7	29.5	0	0	0	0
	H10-8267B1	28.1	28.3	0	0	0	0
102	H10-8267B2	26.9	27.7	0	0	0	0
	H10-8352B	27.5	27.2	0	0	0	0
103	H10-8352C	26.1	28.2	34.9	0	0	0
	H10-8489C	27	26.5	0	0	0	0
104	H10-8528B	26.7	26.2	0	0	0	0
	H10-8737B	27	26.4	0	0	0	0
105	H10-8737C	25.3	29.9	0	0	0	0
	H10-8936B	25.5	28.7	0	0	0	0
	H10-8936C	26.6	29.7	0	0	0	0
106	H10-8936D	27.3	30	0	0	0	0
	H10-8938B	25.2	26.8	0	0	0	0
107	H10-8938C	26.6	27.9	0	0	0	0
	H10-9031C	27.2	27.7	0	0	0	0
	H10-9031D	27.4	28.3	0	0	0	0
	H10-9033B	26.2	28	0	0	0	0
	H10-9033C	26.7	29.1	0	0	0	0
108	H10-9033D	26.6	29.2	0	0	0	0
	H10-9153C	27.1	28.8	0	0	35.3	38.6
	H10-9153F	27.5	29.6	0	0	0	0
	H10-9308B	25.8	27.1	0	0	0	0
	H10-9308C	26.3	27.8	0	0	0	0
109	H10-9308D	27	27.6	0	0	0	38.8
	H10-9310B	28.1	29	0	0	0	0
	H10-9310C	27.5	28.9	0	0	0	0
110	H10-9310D	26.6	27.2	0	0	0	0
	H10-9442C1	26.6	27.2	0	0	0	0
	H10-9442C2	28.8	29.6	0	0	0	0
111	H10-9444B	26.3	25.4	35.5	0	0	0

2.8   The table compares the Cq values of positive cases from the LC480 (480) and Metasin (M) assays.

(The yellow highlighted numbers highlight the discordant results)

Sample	PBGD-480	PBGD-M	CK19-480	CK19-M	MGB-480	MGB-M
12439 B	26.73	30.4	19.31	20.7	17.8	19.1
C	27.16	26.9	21.29	18.1	18.48	16.5
13265 B	25.79	29.2	17.68	19.3	21.16	23.5
15461 B	26.86	28.6	26.86	24.4	26.1	27.9
16837 B	26.02	27.9	23.6	0	0	0
16922 D	25.37	26.5	18.3	0	16.91	20.7
17635 B2	25.78	28.6	27.7	0	33.45	36.2
7867 D	26.09	25.7	26.78	27.7	26.27	23.7
8759 B	25.47	24.5	23.79	23.3	37.14	0
C	23.92	23.9	19.45	19.1	33.9	0
D	23.19	24.5	15.61	17	26.49	30.5
E	24.94	25.1	19.56	18.9	33.9	33.4
F	24.93	24	19.98	18.6	33.81	32.5
G	26.4	24.5	20.47	17.7	34.77	31.4
H	24.75	24.1	18.47	17.1	34.98	30.9
I	25.32	25.1	20	19	27.05	24.1
9041 B	25.86	25.4	0	0	31.56	29.8
10762 D	25.92	26.5	19.6	18.1	16.84	15.3
E	26.19	26.1	18.11	15.5	16.06	15.5
10931 B	26.79	26.2	0	31.7	27.87	25.6
13447 D	26.21	25.4	19.65	16.8	18.51	16.5
E	26.63	24.7	18.97	16.6	17.6	16.7
15131 B	26.11	25.1	0	0	32.78	31.7
16039 B	25.94	24.8	20.43	17.8	20.63	19.8
C	25.53	26.7	19.71	16.4	18.46	16.2

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17502 B	24.84	24.2	17.88	15.6	22.58	21.6
C	26.23	24.2	19.98	16.2	24.86	23
18243 B	26.69	27.4	22.69	22.3	18.8	20.6
C	26.62	24.9	0	20.9	30.03	30.4
745 B	26.59	24.3	23.46	16.3	30.94	22.3
C	26.67	24.3	19.17	16.1	25.03	28.3
925 B	27.24	27.1	22.8	20.5	35.32	34.4

2.9   The table compares the Cq values of the same positive samples run on different days by two different users (day 1 and day 2)

(The yellow highlighted values are discordant)

Sample	PBGD 1	PBGD 2	CK19 1	CK19 2	MGB 1	MGB 2
7867 D	25.3	25.7	27.8	27.7	24.9	23.7
8759 B	24.2	24.5	23.6	23.3	0	0
C	23.5	23.9	19.2	19.1	0	0
D	24.1	24.5	17.4	17	31.8	30.5
E	24.6	25.1	18.9	18.9	34.4	33.4
F	23.5	24	18.7	18.6	33.7	32.5
G	24.2	24.5	17.8	17.7	<mark>32.5</mark>	<mark>31.4</mark>
H	23.6	24.1	17.2	17.1	32.2	30.9
I	24.7	25.1	19	19	25.1	24.1
9041 B	24.8	25.4	0	0	31.3	29.8
10762 D	26.1	26.5	18.3	18.1	16.9	15.3
E	25.6	26.1	16.2	15.5	16.2	15.5
10931 B	25.8	26.2	0	31.7	27.5	25.6
11531 C1	24.4	24.9	16	15.7	17.5	17.5
C2	24.8	25.3	16.6	16.5	16.7	15.9
13447 D	24.8	25.4	17.2	16.8	17.5	16.5
E	24.3	24.7	16.7	16.6	18.2	16.7
15131 B	24.6	25.1	0	0	<mark>33.6</mark>	<mark>31.7</mark>
16039 B	24.4	24.8	18.2	17.8	20.7	19.8
C	26.3	26.7	16.4	16.4	17.2	16.2
17502 B1	23.8	24.2	15.7	15.6	22.3	21.6
B2	23.5	24.2	16.2	16.2	22.4	23
18243 C	24.3	24.9	21	20.9	31.3	30.4
745 B	23.6	24.3	16.4	16.3	29.5	22.3
C	24	24.3	16.3	16.1	24.4	28.3

Commented [SP5]: Why is this not highlighted? Asked same question on hard copy and not answered yet.

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925 B	26.7	27.1	20.9	20.5	35.9	34.4
2344 C	25.4	25.9	0	0	28.9	27.8
3258 A1	23.8	24.6	19.4	27.4	31.7	0
A2	23.7	24.3	19.1	19.1	31.4	30.4
B2	26.1	26.7	0	0	35.8	34.4
C1	25.2	25.8	0	0	0	0
C2	25	25.4	0	0	29.2	28.4
3256 E	25.3	25.7	29	28.4	31.2	29.9

2.10   The table contains the GeneSearch and Metasin extracted RNA concentration of positive nodes and a comparison with the reference gene PBGD Cq value

Sample	PBGD-G	GeneSearch RNA conc (ug/ml)	A260/280	A260/230	PBGD-M	Metasin RNA conc (ug/ml)	A260/280	A260/230
7867	27.6	134	2.094	0.278	25.6	66	2.062	1.269
	27.8	126	2.1	0.426	25.8	128	2.065	0.821
	27.3	128	2.133	0.444	25.7	176	2.047	1.294
	28.3	96	2	0.457	25.6	134	2.03	1.264
	27.8	82	1.952	0.788	26.5	156	2.053	1.2
8759	26.4	212	2.078	0.477	24.5	176	2.095	1.205
	23.8	142	2.029	0.568	23.9	358	2.081	1.421
	23.9	382	2.054	0.946	24.5	344	2.098	1.036
	25.2	270	2.077	0.703	25.1	360	2.118	1.154
	26.6	270	2.077	0.531	24	330	2.089	1.086
	29.1	108	2.077	0.255	24.5	160	2	1.067
	26.4	74	2.056	0.125	24.1	334	2.088	1.21
	26.4	72	2	0.186	25.1	258	2.115	1.229
9041	28.1	76	2	0.528	25.4	52	2.364	5.2
	27.4	118	2.034	0.373	28.4	162	2.25	11.6
9481	27.9	110	1.964	0.585	28.3	126	2.1	0.346
	28.8	116	2	0.362	26.4	158	1.975	0.975
	29.4	78	1.95	0.487	25.8	164	2	0.617
10639	27.8	118	2.034	0.578	25.6	154	2.139	0.542
	27.4	174	2.023	0.654	24.4	206	2.146	0.725
10762	28.6	202	1.942	0.461	26.4	166	2.075	0.456
	28.2	132	1.941	0.496	26.4	182	2.068	0.615
	32.8	120	2.069	0.472	26.5	70	1.944	0.407
	33.9	94	2.043	0.385	26.1	126	2.032	0.521
10931	28.4	166	1.976	0.446	26.2	186	1.979	0.495
11238	32	84	1.909	0.3	27	188	1.958	0.707

11387	27.6	188	1.958	0.566	25.5	172	2	0.729
	27.5	194	2.021	0.366	25.4	256	2.098	1
	27.6	178	1.978	0.503	25.8	246	2.016	0.904
11531	29	128	2.065	0.176	25.7	190	1.979	0.864
	28.2	136	2.061	0.442	25.5	202	1.98	0.808
	32.9	60	1.875	0.065	25.3	172	2.048	0.614
	28.5	202	1.98	0.234	28.2	264	2.031	0.663
12395	28.6	140	2.059	0.833	26.1	236	2	0.559
	32.9	18	2.25	0.134	28.2	14	1.75	0.14
	32.6	98	2.042	0.516	25.4	166	2.128	0.5
	33.3	162	2.025	0.596	24.7	284	2.119	1.06
13742	29.9	66	1.833	0.589	25.7	118	2.034	0.476
	29.5	162	2.025	0.435	26.3	158	2.026	0.731
14732	29.2	136	1.943	0.428	26.3	132	21.129	0.512
15131	25.7	174	2.071	0.6	25.1	218	2.057	0.58
	26.3	150	2.027	0.475	24.8	200	2.041	0.676
	33.4	102	1.889	0.927	25.4	114	1.966	0.438
	26.1	176	2	0.423	25.3	216	2.077	0.837
15664	25.7	202	2.02	0.754	26.1	280	2.09	1.061
	26.7	210	1.981	0.991	26	226	2.055	0.856
15813	26.3	172	2.048	0.677	25.8	212	2.12	0.862
	26.1	212	2.038	0.716	25.3	270	2.143	1.08
16039	25.7	268	2.03	1.196	24.8	366	2.056	1.123
	25.7	244	2.033	1.326	26.7	322	2.118	1.006
	26.5	112	2	0.519	24.8	136	2.125	0.482
	29.1	48	2.182	0.2	25.7	100	2.083	0.427
17502	25.9	210	2.1	0.538	24.2	32	2	0.107
	26.1	190	2.065	0.397	24.2	214	2.14	0.594
18243	27.6	292	2.056	0.619	27.4	300	2.113	0.456
	27	206	1.981	0.433	24.9	214	2.098	0.673
18472	26.8	94	1.958	0.635	25.8	176	2.047	0.978
	27.8	74	2.056	0.303	26.2	128	2.065	0.719

745	24.8	150	2.083	0.577	24.3	174	2.071	0.584
	26.4	150	2.027	0.387	24.3	200	2.174	0.641
925	27.5	100	1.923	0.549	27.1	156	2.053	0.433
1443	26.1	106	1.963	0.515	26	200	2.041	0.654
	26.4	134	2.03	0.578	25.1	134	2.161	0.598
	26.5	144	2	0.673	25.4	112	2	0.326
1445	25.5	168	2.1	0.382	24	248	2.102	0.992
	25.8	142	2.152	0.514	24.2	230	2.13	1.095
	26.3	150	2.027	0.339	25.2	162	2.077	0.771
	25.9	160	2.105	0.39	25.1	150	2.083	1.027
1643	25.8	148	2.114	0.409	25.2	216	2.077	0.885
	26.3	122	2.103	0.462	25.4	204	2.125	0.607
	28.4	58	1.933	0.358	26.7	86	2.048	0.589
2344	26.5	122	2.103	0.61	25.6	208	2.039	0.945
	27.5	140	2.059	0.519	25.9	164	2.103	0.752
	30	96	2.087	0.279	34.4	170	2.073	0.503
2620	26.3	102	2.04	0.26	25.3	126	2.1	0.525
3258	24.1	88	1.193	0.647	24.6	218	2.137	0.553
	24.8	148	2.056	0.13	24.3	296	2.085	0.841
	26.9	118	1.844	0.418	26.7	72	2	0.396
	27.7	118	1.903	0.711	25.8	94	2.043	0.263
	27.8	180	1.957	1.023	25.4	130	1.97	0.684
3256	26.4	116	2	0.227	26.3	198	2.063	0.892
	25	186	2.114	0.296	24.8	198	2.106	0.917
	25.4	84	2	0.082	24.4	254	2.016	1.067
	26.5	56	2.333	0.067	25.7	202	2.104	0.759
3820	26.3	64	2.133	0.323	26	156	2.108	0.549

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STANDARD OPERATING PROCEDURE FOR METASIN

Title	**PROCEDURE FOR BREAST SENTINEL LYMPH NODE ANALYSIS USING THE METASIN BLN ASSAY**
Code	
Version Number	
Date of Implementation	
Review Date	
Replaces	
Location	
Author: **Signature:** **Date:**	
Authorised by: **Signature:** **Date:**	
Copy No:	

Date Reviewed	Final review	Reviewed by	Changes	Next |Review

NAME	DATE SOP READ:	SIGNATURE:

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1.   Clinical Relevance / purpose of examination

The Metasin BLN Assay is a qualitative, in vitro diagnostic test which enables rapid detection of > 0.2mm metastases in nodal tissue removed from breast sentinel lymph node biopsies. The results from this test can be used to guide the intra-operative or post-operative decision to remove additional lymph nodes.

2.   Principle of examination

The Metasin BLN Assay is a real time reverse transcriptase polymerase chain reaction (RT-PCR) assay that detects the presence of breast tumour cell metastasis in lymph nodes through the detection of gene expression markers Mammaglobin (MG) and Cytokeratin (CK 19). These markers are present in higher levels in breast tissue, but not in nodal tissue. The Cepheid SmartCycler® system is used to generate expression data for these genes of interest.   Expression results are then applied against predetermined criteria to create a qualitative result (Positive, Negative or Invalid).

To standardise the distribution of tissue sampling, lymph nodes are divided into sections and alternating sections are combined and processed using the Metasin BLN Assay. The remaining sections are used for routine histology. Each lymph node is individually processed. The nodal tissue is homogenised to release RNA molecules. RNA is purified from the tissue homogenate and RT-PCR is performed.

The real time RT-PCR reaction is performed in a homogeneous, one-step, enclosed reaction. Three gene markers (MG, CK 19 and an internal control gene [PBGD]) are included in the reaction. Each gene marker is detected using fluorescent molecules with different excitation and emission wavelengths. Following each temperature cycle of the PCR reaction the fluorescence is measured and amplification of the gene marker is detected through increased fluorescence. The cut-off (Ct) value is determined when

the fluorescent signal exceeds a pre-defined threshold limit. If the Ct value for either or both gene markers is less than the cut-off value then the sample is positive.

3.   Related procedures / SOPs

Breast Sentinel PCR Stock Check

Sentinel Lymph Node Biopsy

Pipette Proficiency Record

4.   Hazards and safety precautions

All biological specimens and reagents coming into contact with the specimen(s) are considered biohazardous and should be handled with care.

Prior to and after performing PCR analysis, bench surfaces must be wiped down with Tristel. Buffer RLT spills to be wiped dry using RNase-free water before using Tristel.

Microbial contamination of reagents must be avoided.   RNA is susceptible to degradation it is therefore important to maintain RNase free conditions. All personnel should follow safety precautions and wear the appropriate personal protective equipment (laboratory coat, gloves) at all times. Gloves should be changed between specimen preparation and PCR amplification/detection activities to avoid contamination. A fume hood should be used for preparing the sample preparation kit reagents, particularly when using β-mercaptoethanol. A face shield may be worn when making up and handling the homogenisation buffer.

The homogenisation buffer (Buffer RLT Lysis buffer) contains guanidine thiocyanate. This is associated with the following risk and safety phrases: Harmful by inhalation, in contact with skin and if swallowed (R20/21/22), contact with acids liberates very toxic

gas (R32), in case of contact with eyes rinse immediately with plenty of water and seek medical advice (S26), if swallowed seek medical advice immediately and show label or container (S46).

Guanidine thiocyanate can form highly reactive compounds when combined with bleach. If spilt, use a suitable laboratory detergent and water to clean spills followed by bleach solution if decontamination is required.

Buffer RW1 (Wash Buffer 1) contains 10% ethanol (Flammable, R10).

Buffer RPE (Wash buffer 2) contains sodium azide. Harmful if swallowed. After contact with skin, wash with plenty of soap.

Some of the reagents contain Proclin 300 preservative. Symptoms of overexposure to Proclin 300 may include irritation of skin, eyes, mucous membranes and upper respiratory tract.

After running the amplification reaction, to avoid amplicon contamination **do not** open sample or control tubes under any circumstances in the PCR area. After each run, clean the work areas (including applicable equipment) using Tristel.

Avoid contaminating reagents with bleach. Contamination will cause erroneous results.

5. Specimen requirements and means of identification

Before preparing the lymph node, set up a fresh disposable cutting board, put a fresh blade on the scalpel and use a fresh pair of gloves. **Change gloves, scalpel blades, forceps, and cutting surface between lymph nodes – this is essential to minimise sample cross-contamination.** For further details on slicing lymph node please refer to LP Sentinel Lymph Node Biopsy.

- Care must be taken to minimise contamination of the lymph node with breast or primary tumour tissue, as this may lead to a false positive result.
- Lymph node tissue should be received in an appropriately labelled specimen pot. For transport purposes these are usually placed inside a larger specimen pot.
- To prepare working Buffer RLT, add 450µl β-mercaptoethanol (β-ME) to one bottle of Buffer RLT and mix well. Reagent is stable for 30 days after β-ME has been added, make a note of expiry date on bottle. **Warning! B-ME is toxic, wear appropriate PPE and dispense in a fume hood.**
- Label Buffer RW1 as 'Wash Buffer 1'.
- To prepare Buffer RPE, add 44mL absolute 200-proof ethanol to the Buffer RPE concentrate and mix well, label this bottle as 'Wash Buffer 2'. Reagent is stable for 30 days when stored at ambient temperature after ethanol addition, make a note of expiry date on bottle.
- To prepare 70% ethanol, add 7mL 200-proof molecular biology grade ethanol to 3mL of nuclease-free water. **Ensure solution is mixed well prior to use** – incomplete mixing prior to use may cause erroneous results.
- The lymph node should be prepared as soon as possible to minimise RNA degradation.
- Each lymph node should be processed as a separate specimen. Tissue is stable for 45 minutes at room temperature after removal from the patient.
- Tissue homogenate and purified RNA should be stored in the -70ºC freezer and have a diagnostic shelf life of 21 days and 9 weeks respectively. Please log case number and date of preparation, disposal date on chart on Freezer log folder.

6.   Equipment and special supplies

Cepheid SmartCycler® Diagnostic System including:
- I-CORE™ blocks

- Mini-centrifuge
- Plastic sample racks
- Computer (incl. monitor from Cepheid)
- Tube puller
- Cepheid 25μL reaction tubes for Cepheid SmartCycler® (900-0003)

- Printer
- 1.7 mL polypropylene microcentrifuge tubes, certified DNase- and RNase-free (COSTAR 3621)
- 1.5mL microcentrifuge tubes (FISHER SCIENTIFIC FB56089)
- 0.5ml microcentrifuge tube
- Vacuum System (KNF LAB LABOPORT® NF840 FT.18)
- Qiagen® VacConnectors (19407)
- Qiagen® VacValves (19408)
- Vacuum source (capable of drawing -800 to -1000 mbar)
- Qiagen® QIAvac 24 Plus Vacuum Manifold (19413)
- Qiagen® Vacuum Regulator (19530)
- Omni Homogenizer (QLH-02)
- Omni Adaptor
- Omni Tip™ Disposable Probes for Omni Homogenizer (OMNI INTERNATIONAL 30750)
- 8mL polypropylene culture tubes for homogenization (VWR 211.0074)
- SAV-IT closures (FISHER BRAND 02-707-10)
- 14mL polypropylene tubes for homogenisation
- Disposable forceps (TWD DF8088N)
- Scale
- Weighing boats (FISHER BRAND 08-732-113)

- Calibrated pipettors:
- Finnpipette 1000μl (THERMO SCIENTIFIC)

- Finnpipette F2 10 - 100µl (THERMO SCIENTIFIC 4642070)
- Finnpipette F2 1 - 10µl (THERMO SCIENTIFIC FJ46041)

- Pipette-Aid
- Aerosol resistant, DNase- and RNase-free tips:
- Pipettor tip sureone filter sterile rack micropoint tip 0.1 - 10µl (FISHER SCIENTIFIC FB78100)
- Pipettor tip sureone filter sterile rack bevelled tip 10 - 100µl (FISHER SCIENTIFIC FB78112)
- Pipettor tip sureone filter sterile rack micropoint tip 100 - 1000µl (FISHER SCIENTIFIC FB78112)
- Pipettor tip finntip sterile 2 – 10mL (FISHER SCIENTIFIC PMP-103-260E)
- Pipettor tip finntip sterile
- Cepheid centrifuge (700.2378)
- Centrifuge
- Vortex (IKA® Vortex Genius 3)
- Disposable scalpels (SWANN-MORTON SCA-310-030)

7. Reagents, standards and internal quality control materials

- Qiagen RNeasy Mini Kit (QIAGEN 74104)
- LightCycler 480 RNA Master Hydrolysis Probes (ROCHE 04 991 885 001)
- β-Mercaptoethanol (CALBIOCHEM 44203)
- 200-Proof (Absolute) Ethanol, molecular biology grade (SIGMA ALDRICH 245119-1L)
- Water sterile non-DEPC treated (nuclease-free) (FISHER BIOREAGENTS VX10977049)

8. Calibration

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Before commencing with processing check vacuum pressure of vacuum manifold is between 800 – 1000mbar.

Pipettes should be calibrated regularly and pipette proficiency tests performed. A sticker should be attached to the pipette with date of calibration and initials of the individual performing the test. Calibration results should be kept in the individuals training manual. Please refer to pipette competency forms LF730048.

9. Procedure / instructions for performance of the examination

Prior to proceeding, please follow the Assay Preparation Check List.

The procedure is divided into 3 sections:
1. **Lymph Node Tissue Homogenisation & RNA Extraction**
2. **RNA Amplification Setup**
3. **SmartCycler® Operation**

The check list and all procedures are given below as per manufacturer's instructions, for further details please refer to documentation under reference section of SOP.

Metasin BLN Assay preparation Checklist

Sentinel Node Dissection Area:

- Turn Scale on
- Wipe workspace with Tristel
- Check supply of weigh boats
- Check supply of scalpels
- Check supply of forceps
- Disposable cutting boards
- Transparent Ruler
- Notepad, Pen, Marker
- Formalin Pots

Sentinel Node Homogenisation Area:

- Check supply of homogenisation probes
- Label homogenisation tubes
- Ensure adequate supply of working homogenisation buffer
  - Prepare more if required by adding 450µl of β-mercaptoethanol to a 45ml bottle of Buffer RLT.
  - Note on the bottle the 30day expiry date from the day β-mercaptoethanol is added to Buffer RLT
  - Prepare one 14ml homogenisation tube with 5ml of Buffer RLT
  - Prepare one 8ml homogenisation tube with 2ml of Buffer RLT

RNA Extraction Area:

- Ensure adequate supply of 70% ethanol:

Preparation of 70% Ethanol		
Final Desired Volume	Volume 200-proof Ethanol to Add	Volume Nuclease Free water to add
50ml	35ml	15ml

10ml	7ml	3ml

- Attach VacValves and clean VacConnectors to manifold
- Check Qiagen RNeasy Mini Kit to ensure adequate reagent quantities and volumes:
    - RNA spin columns
    - Buffer RWI (Wash Buffer 1)
    - Ensure that adequate supply of *working* Buffer RPE (Wash Buffer 2) is prepared
        - Prepare more if required by adding 44ml of 200-proof ethanol, molecular biology grade to a new bottle of Buffer RPE concentrate
    - Label 2 sets of 1.5ml centrifuge tubes per sample
    - Set centrifuge/confirm centrifuge settings (0.5min, >10,000 rpm)
    - Confirm vacuum and manifold operation by turning vacuum on and verifying vacuum is adequate (~800 to ~ 1000mbar)
    - Set 200µl pipette to 50µl, check tip supply

Amplification area:

- Label cepheids, remember to include positive control (PC) and negative control (NC).
- Set up SmartCycler® as follows:
    - *Turn on SmartCycler® and computer, log on to the SmartCycler® Dx software.*
    - From the CREATE RUN Screen, select METASIN BLN Assay
    - Enter Run Name as date (DD-MM-YY) and Histology number; enter the PCR Kit lot # & expiration date (YYYY-MM-DD).

Preparation for RNA AMPLIFICATION PROCEDURE

Start this procedure once the sentinel lymph nodes have arrived in the department.

NB. This procedure is based on having up to 4 nodes with positive and negative control. For more nodes additional primer/probe mix (BROWN tube) and activator (WHITE tube) will be required.
e.g. 5 nodes = 2 BROWN tubes

Use a **fresh tip** for each step below:

1. Use fresh gloves.
2. Ensure cepheids are labelled correctly and placed in the blue Cepheid rack on the cool block.
3. Thaw adequate supply of primer/probe mix (BROWN tube) and activator (YELLOW tube aliqouts), positive and negative controls.
4. Pulse spin all thawed reagents before use and place these in the cool box with the enzyme mix (RED tube).
5. Add 57µl of enzyme mix into the mouth of the brown tube. Discard tip.
6. Add 10µl of activator into the mouth of the brown tube. This constitutes the Master Mix.
7. Mix thoroughly by flicking the base of tube 5 times, then pulse spin for 6 seconds.
8. Aliquot 21µl of the Master Mix into each of the cepheids.
9. Add 4µl of Negative Control to "NC" tube; discard tip; close tube.
10. Add 4µl of Positive Control to "PC" tube; discard tip; close tube.
11. Spin the Control Cepheids and place in relevant ICORs.
12. Leave other Cepheids on rack until sample RNA has been extracted (see RNA AMPLIFICATION PROCEDURE - p10)
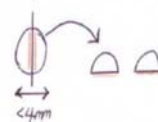
Lymph Node Dissection

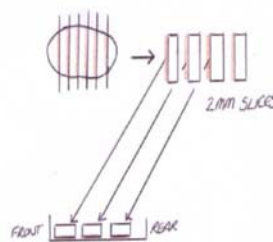*These nodes are sliced as per protocol and all slices are processed histologically.*

1: Use cut up board and blades provided, change board and blades for each node.

2: Weigh LN in weigh boat after removal of fat & record weight in mg.

3. Record dimensions of node (mm).

  The minimum amount for Metasin processing is 3mg (0.003g) if the nodes weigh less than this in total then DO NOT GIVE SAMPLE TO METASIN as the procedure at present is not licensed for whole node processing, keep all tissue and process using conventional histological technique.

3: If LN is less than 4mm, equally bisect and place histology slice downwards in TURQUOISE cassette between blue foams, give other slice for Metasin processing.

4: if LN is greater than 4mm, bread slice every 2mm along the short axis (not transversely) and placed with non opposing cut edges down.

5: Alternate slices to be given for Metasin processing,

Example of node cut-in based on node size

Node A   Node B

3.0 mm   12.0 mm

2mm slices

FRONT   REAR
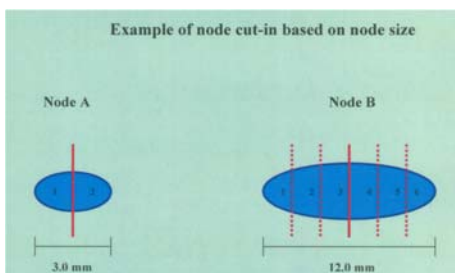
slice number needs noting on form. (1, 3, 4… or 2, 4, 6…) The BMS will decide which slices will be sent for Metasin.

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6. Place the slices for histology weight boat labeled with node number and place in fume hood. Pour some formalin over the tissue to fix them. On completion of Metasin assay, transfer the slices into <u>Turquoise</u> CASSETTES maintaining the same orientation (between blue foams) no more than **two slice** per cassette are placed from front to back as cut, record details on adhesive labels available in the BRSN folder. Attach the labels to the back of specimen request form for the BRSN.

Cassettes labelled BRSN are placed into the routine refix pot and processed the following day on a routine programme, there is no requirement for these to be placed on an overnight breast run unless the nodes are particularly fatty and require longer processing via the breast programme.

Lymph Node Tissue Homogenisation

Work under fume hood in well ventilated area

1. Determine weight (mg) of tissue to be used in assay.
2. Mince the slices of tissue using scalpel into smaller fragments.
3. Determine the amount of Buffer RLT and appropriate sized tube for each sample according to table below:

Tissue Weight (mg)	Buffer RLT (ml)	Tube Size (ml)
3-149	2	
150-199	3	8
200-249	4	
250-299	5	
300-349	6	
350-399	7	
400-449	8	14
450-500	9	

500-550	10	
>550	See note	

NOTE: *Tissue weighing greater than 550mg will not be adequately homogenised. Divide the tissue into equivalent parts prior to homogenisation and proceed as if they were individual specimens.*

4. Transfer tissue into the homogenisation tube.
5. Add buffer RLT to homogenisation tube.
6. Homogenise tissue completely.

METASIN RNA PURIFICATION

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1. In a 1.5ml tube, mix 400µl of homogenate with 400µl of 70 % ethanol and vortex for 10 seconds.
2. Determine the amount of homogenate:ethanol mix to add to column:

Tissue Weight (mg)	Homogenate:Ethanol Mix (µl)
3-39	700
40-49	500
50-59	400
60-69	350
70-79	300
80-89	250
90-99	225
≥100	200

3. Add the indicated volume to the column
4. Apply vacuum. Allow the entire sample to filter completely.
5. Close VacValves. Add 700µl of Buffer RW1 (Wash Buffer 1) to the column and filter

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6. Close VacValves. Add 700µl of Buffer RPE (Wash Buffer 2) to the column and filter

7. Close VacValves. Remove each column from the Vacuum Manifold and place into the round-bottom collection tube supplied with the column.

8. Centrifuge tube containing the column for 30 seconds at >10,000RPM

9. Discard collection tube. Remove the Column and put into new 1.7ml centrifuge tube.

10. Add 50µl of RNase-free water directly to the filter membrane of the column.

11. Centrifuge for 30seconds at >10,000PRM

12. Ensure 1.7ml centrifuge tube is labelled. Discard the column. Approximately 50µl of eluted RNA solution will be contained in the collection tube.

RNA AMPLIFICATION PROCEDURE

Use a **fresh tip** for each step below:

1. Add 4µl purified RNA to pre-prepared cepheids; discard tip; close tube.

2. Repeat step 1 for each additional RNA sample.

3. Ensure caps are snapped.

4. Spin all Cepheid tubes in the SmartCycler® centrifuge for 6 seconds.

5. Discard the BROWN and WHITE tube after use & return reagents to freezer.

6. On the SmartCycler® screen, add the number of specimens to be tested where prompted. Select APPLY.

7. For patient specimens, change "Spec" in Sample ID column of Site Table to node ID (e.g B, C, D) if multiple nodes are being run from the patient.

8. After all specimens have been placed in the SmartCycler®, select START RUN.

9. When prompted, re-enter password.

10. At the end of the test (approx. 28 mins) in the VIEW RESULTS Screen, click on Results Table and check control results to determine if the run was valid. Both qualitative and quantitative results are provided.

11. Phone relevant theatre and give the results to theatre staff member. Ensure name of staff member is noted on the request form and ensure they are able to reiterate the results.

12. Both qualitative and quantitative results must also be noted on the request form.

MASTER MIX PREPARATION/RESULTS TEMPLATE

Qiagen Lot No:_____

Roche Lot No: _____

		Date:	
S _____-_____		**BMS:**	
PREPARE MASTER MIX IN BROWN TUBE **Lot No:**			**ADDED**
ENZYME MIX **(RED TUBE)**	**57µL**		√
ACTIVATOR **(WHITE TUBE)**	**10µL**		√
Flick tube (X6 & Pulse spin (6 secs)			√
MASTER MIX (LN + 2) **Max. 4N/brown tube**	**21µl**	**/** **Cepheid**	√
-VE CONTROL	**4µl**		√
+VE CONTROL	**4µl**		√
RNA	**4µl**		√
CUT OFF VALUES: **CK19 ≤32** **Macro <25)**	**+VE if** **(**		**MGB≤32.3** **(Macro** **<26)**

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Node	Weight (mg)	Slices	Metasin Weight	PBGD		CK19		MGB	
				RESULT:					

Results Given to:

10. Limitations / pitfalls of the examination (including interference, cross reactions and reportable intervals.

- It is important to avoid contaminating lymph nodes with breast tissue. Breast tissue can yield false positive results.

- Presence of excess fat surrounding the lymph node tissue decreases the sensitivity of the test and can lead to an "Invalid" result.

- Fixing the tissue in formalin prior to testing will result in an "Invalid" result.

- Pooling of separate lymph nodes must not occur as it may result in a loss of test sensitivity and cause erroneous results.

- Processing at ambient temperatures above 30°C may lead to loss of test sensitivity.

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- Tissue <50mg in weight may produce a higher Invalid test rate with the Metasin BLN Assay.

- The Metasin BLN assay has been designed to work with sentinel lymph node procedures yielding no more than 6 samples per run. 2 runs may be performed simultaneously or sequentially accommodating a total of 12 samples per SmartCycler.

- The assay is not designed for use with formalin-fixed tissue.

- **Accurate pipetting is essential for optimal results.**

11.   Recording, calculation and transcription of results

All results are reported by a pathologist. Once the run is completed (approximately 24 minutes) the software displays the result as positive or negative, this information is phoned through to theatre staff together with the quantitative result (e.g. macro-/micro-metastases) by a qualified BMS.

If a run fails, testing may be repeated dependent on the mode of failure.

External control failures: If run is invalidated, the assay can be repeated using residual RNA sample from patient lymph node(s) and external controls from the assay test kit.

Internal control failures: If sample fails due to all markers (including internal control) being negative for a given patient (from one or more lymph nodes), RNA can be re-purified from the homogenate(s) and assay repeated.

NB. The tissue homogenate and purified RNA are stable up to 60 minutes at ambient temperature.

12. Internal quality control procedures and other criteria for which acceptability are judged

External controls are provided for MG and CK19 (positive control) and for PBGD (negative control). These must be included in each run. The controls monitor the quality of the reagents and the instrument performance thereby reflecting on the performance of the assay.

The internal control consists of detection of mRNA from a house keeping gene (PBGD) always expressed in lymph node tissue, and thereby acts as a control against false negative results. This control monitors sample quality, sample preparation and the RT-PCR reaction. Extremely high expression of the cancer markers (MG and CK19) may inhibit detection of the internal control. In this circumstance, assays in which one or both cancer markers are positive in at least one lymph node are considered valid, regardless of the internal control result.

13. Reporting reference limits including alert/critical results

NB. Ensure validity of Positive and Negative Controls before giving results to pathologists.

Results from runs with one or more Invalid controls **must** be repeated and **must not** be reported.

14. Performance criteria

The cutoff values are specific for each marker. External control values must be valid. Samples with Ct values less than or equal to one or both of the cutoff values for MG or CK19 are considered positive. If the MG and CK19 Ct values are above their cutoffs the Internal Control Ct value must be below its cutoff for MG and CK19 to be considered negative. If the MG and CK19 Ct values are negative and the Ct value for the Internal Control gene is greater than or equal to its cutoff, then the result for that sample is considered "Invalid".

The cutoff Ct values for the markers are as follows: CK19 ≤ 32, MG ≤ 32.3 & Internal Control < 36.

If result is "Valid" the Patient Results section shows the test result to be "Positive" or "Negative". The result is per patient not per lymph node. If at least one lymph node being tested for a patient is positive for either marker, the Patient result is "Positive", regardless of results obtained with other samples from that patient. Conversely, if Patient result is not "Positive" and the internal control result from one or more of the patients' lymph nodes is "Invalid", then the Patient result is "Invalid".

15.   Result Reporting and authorisation of reports

The pathologist assigned to the case will be informed of the result and the result will be noted on the patients histology form.

16.   References

- Cepheid SmartCycler® Diagnostic Operator manual
- QIAGEN RNeasy Mini Kit insert
- ROCHE LightCycler 480 RNA hydrolysis kit insert
- *Int. J. Mol. Sci.* **2013**, *14*, 12931-12952; doi:10.3390/ijms140712931