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**MOLECULAR DETECTION OF MELANOMA NODAL
METASTASES**

VIRGINIA DAVIDS, MBChB (UCT)

**Thesis presented for the degree of
Master of Science in Medicine
In the Department of Human Biology,
Faculty of Health Sciences
University of Cape Town**

May 2002

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DECLARATION

I, **Virginia Davids**, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work or any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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May 2002

DEDICATION

To my precious husband and best friend,
and my wonderful mom who passed on from this world during the
production of this thesis, and whom I miss so much.

University of Cape Town

Be completely humble and gentle; be patient, bearing with one another in love. Make every effort to keep the unity of the Spirit through the bond of peace

Ephesians 4: 2-3

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LIST OF ABBREVIATIONS

bp	base pair
CTA	cancer/testis antigen
cDNA	complementary DNA
CMC	circulating melanoma cell
ddH ₂ O	double distilled water
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxynucleotide 5'-triphosphate
ECLND	elective complete lymph node dissection
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
H&E	haematoxylin and eosin
IHC	immunohistochemistry/immunohistochemical
JT	Jurkat T
MIA	melanoma-inhibitory activity
mRNA	messenger RNA
OD	optical density
PBGD	porphobilinogen deaminase
PBS	phosphate-buffered saline
PCS	pigment cell-specific
RLN	regional lymph node
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute 1640 medium
RP5	RPMI medium plus 5% fetal calf serum
RT	reverse transcription
RT-PCR	reverse transcription-polymerase chain reaction
SL	sentinel lymphadenectomy
SN	sentinel lymph node
TBE buffer	tris-base/EDTA buffer
TEN buffer	Tris-HCl / EDTA / NaCl buffer
τRNA	total RNA
WLE	wide local excision

ABSTRACT

The successful management of patients presenting with various solid malignancies, including cutaneous melanoma, depends largely on the early identification of metastatic spread from the primary tumour. The application of lymphatic mapping and sentinel lymph node (SN) biopsy to primary melanoma patients has recently revolutionised the ability to identify those melanoma patients who already have subclinical/microscopic regional lymph node metastases at initial presentation. Accurate histopathological evaluation of the surgically removed SN is vital, as this will determine subsequent management decisions. However, this standard of evaluation carries a significant false-negative risk, even with additional serial sectioning and immunohistochemistry. Furthermore, histopathological SN analysis is extremely labour-intensive and costly. Molecular detection of SN metastases is significantly more sensitive than histopathology and facilitates the cost-efficient analysis of the entire SN using multiple markers. RT-PCR evaluation of the SN is therefore being investigated as an alternative to histopathology. Unfortunately, a lack of marker specificity currently limits the molecular evaluation of SN tissue from becoming clinically applicable.

The aim of this study was to develop a practical and reproducible multi-marker RT-PCR assay, with the emphasis on achieving maximum specificity for the detection of melanoma nodal metastases. A novel protocol for the efficient homogenisation of nodal tissue was developed, with clinical applicability as the objective. Three pigment cell-specific (PCS) markers, namely tyrosinase, Pmel-17 and MART-1, as well as one cancer/testis antigen (CTA), namely MAGE-3, were selected. The PCR assay for each marker was optimised so as to avoid detecting 'unwanted' transcripts in normal control nodes. Small nodal nevi are present in approximately 8.5% of melanoma-draining SNs that do not contain metastases. These neval cells are another potential cause of RT-PCR false-positives when the PCS markers (co-expressed by melanoma and neval cells) are used. However, this risk cannot be determined or eliminated since appropriate control tissues (i.e. normal nodes containing nevi) are essentially unavailable. The expression of the selected molecular markers in nodal melanoma cells and skin melanocytes was therefore compared to confirm the role of the cancer/testis antigen to differentiate between nodal nevi and melanoma cells in the SN. The sensitivity and detection rates of each marker for melanoma metastases were assessed using the optimised RT-PCR assays.

High specificity was achieved for each marker by optimising the PCR cycle number such that 'unwanted' transcripts remained undetectable in appropriate control tissues. Tyrosinase was 100% specific at 40 PCR cycles, MAGE-3 and MART-1 at 35 PCR cycles and Pmel-17 at 30 PCR cycles. Tyrosinase proved to be the most sensitive marker, detecting 10 melanoma cells in 0.1 g of nodal tissue. Although limited numbers of cell lines and melanoma-involved nodes were assessed for marker detection rate, the preliminary results indicate that any one of the markers alone provide the same detection rate as the multi-marker assay. Good reproducibility of the entire nodal processing and RT-PCR protocol for the detection of very low numbers of melanoma cells in nodal tissue was shown. MAGE-3 was found to be the only marker that is not expressed by melanocytes, whilst the other three markers might contribute to false-positive results when analysing melanoma-draining SNs.

In conclusion, this study has shown that a highly specific and sensitive multi-marker RT-PCR assay for the detection of nodal metastases is achievable by careful and systematic optimisation of essential assay parameters. However, the use of the PCS markers carries a potential risk of false-positives due to the presence of nodal nevi in melanoma-draining SNs. Likewise, the use of breast epithelial markers for the detection of breast metastases in SNs will carry a similar risk of false-positives, due to the presence of benign epithelial inclusions in breast cancer-draining SNs. In light of this, it is recommended that more emphasis be placed on the development of a panel of CTAs, which will be useful to detect melanoma as well as other carcinomatous metastases. Once such a panel has been shown to provide specific and optimum detection of melanoma SN metastases, the PCS markers can be excluded to ensure a zero false-positive rate. In addition, the CTAs are more likely to have prognostic value than the PCS markers, since the former are essentially involved in the metastatic process, while the latter are involved solely in the pigmentation pathway.

CHAPTER 1**LITERATURE REVIEW****1.1 INTRODUCTION**

Cutaneous malignant melanoma arises from mutations in epidermal melanocytes, often within a pre-existing mole/nevus.^{1, 2} It is one of the most lethal of all human cancers^{3, 4} since the primary tumour cells usually metastasise at a very early stage of disease. Although patients with no clinical evidence of metastatic spread at initial presentation (i.e. clinically-localised melanoma) may seem to be cured by wide local excision (WLE) of the primary tumour, many of them will nevertheless go on to develop melanoma recurrences.

The above observations imply that a significant proportion of patients who present with clinically-localised melanoma will in fact already have sub-clinical/occult metastatic spread. These metastases are not detected by routine clinical investigations (physical examination^{5, 6} and imaging analyses⁷⁻⁹), because these techniques are limited by a sensitivity that is dependent on a critical minimum tumour mass. Unfortunately, by the time melanoma metastases become clinically-detectable, the prognosis will have decreased significantly (5-year survival rate of less than 60%).¹⁰

The association between the appearance of clinically-detectable metastases and a significant decrease in survival rate has long been recognised. This has prompted investigators to search for prognostic variables that might accurately categorise (stage) those patients with clinically-localised melanoma into different risk groups for occult metastatic disease. Other investigators have focussed on sampling various target tissues in attempts to directly determine whether metastasis has occurred or not. The resultant advances in melanoma patient staging have enabled the attending clinician to provide the melanoma patient with a more accurate prognosis, and to initiate earlier, more effective treatment of those patients who are at high risk of (or already have) occult metastases. The improved detection of occult metastases and the subsequent management of these patients has become particularly important during the last decade: This is because many more patients are now presenting with clinically-localised melanoma, this mainly owing to successful public education campaigns regarding suspicious skin lesions.^{11, 12}

The most significant recent advancement in the accurate staging of melanoma patients has been in the detection of occult metastatic spread to the regional lymph nodes (RLNs), i.e. the group of lymph nodes that directly drains the primary tumour. This is achieved by directly, yet conservatively sampling the RLN basin via sentinel lymphadenectomy (SL) and histopathological evaluation of the sentinel node (SN). SL now allows melanoma patients with no clinical evidence of RLN involvement to undergo histopathological evaluation of the RLN basin. This is particularly advantageous because metastatic melanoma spread tends to occur most commonly to the RLNs,¹³⁻¹⁵ and accordingly, the status of the RLNs has been shown to be a very (if not the most) powerful prognostic indicator.¹⁰ Furthermore, adjuvant therapy with Interferon gamma has recently been shown to significantly improve prognosis for patients with occult RLN metastases,¹⁶ and it has also been confirmed that removal of RLNs harbouring occult metastases does improve the long-term survival rate.^{17, 18}

However, despite the significant advances made in being able to obtain such valuable tissue, the evaluation of this tissue remains sub-optimal. Therefore, the molecular analysis of SN tissue as a more sensitive and cost-effective alternative to histopathology, has become a topic of intense investigation.

The following sections provide a review of the historical developments and improvements in the staging and management of patients with clinically-localised melanoma. Developments in the histopathological and molecular detection of melanoma nodal metastases are reviewed, and the strengths, limitations and clinical significance of these techniques are evaluated. The review will also highlight how multidisciplinary interaction has been, and still is, important to develop the most accurate staging system for melanoma patients. The advances achieved for melanoma have far-reaching implications in that these are likely to be applicable to patients with breast,^{19, 20} oesophageal²¹ and prostate²² cancers, since the status of the RLNs in these patients has also been shown to be the most powerful predictor of survival.²³

1.2 HISTORICAL DEVELOPMENTS IN THE IMPROVED STAGING OF PRIMARY MELANOMA PATIENTS

1.2.1 Elective complete lymph node dissection

The use of the elective complete lymph node dissection (ECLND) procedure, which involves the surgical removal of clinically-uninvolved RLNs, has been one of the most debated controversies in surgical oncology for decades.²⁴ As far back as the late 1800's, proponents of ECLND have justified its therapeutic potential as follows: Metastatic spread of melanoma cells from the primary tumour seems to occur first via the lymphatics to the RLNs, and these nodes can act as a temporary barrier to metastasising cells,²⁵ which spread only later to distant sites.^{2, 13, 14, 26} Thus an intermediate stage in the natural course of the disease, when metastatic spread is restricted to the RLNs (i.e. locoregional disease), is thought to occur. Hence ECLND could be justified as it might remove residual tumour deposits that would otherwise be the source for later distant spread.^{27, 28}

More recently, the benefit of removing RLNs that harbour occult metastases has been confirmed: The 5-year survival rate for patients who, having undergone ECLND are found to have histopathological evidence of occult nodal metastases, is 60%. This is to be compared to a 5-year survival rate of only 40% for those patients who have their RLN metastases removed only once these have become clinically-detectable (i.e. via a therapeutic lymph node dissection).^{10, 15, 29-31} This poor survival rate is explained by the finding that once RLN metastases become clinically-detectable, 70-85% of these melanoma patients will already harbour widespread distant metastases, with little hope for curative surgery.²⁷ Thus, the removal of occult nodal metastases seems to provide a significant therapeutic benefit by having the potential to remove all residual disease.

In addition to the therapeutic role of ECLND, the presence or absence of occult RLN metastases has been shown to be of significant prognostic value: As mentioned above, those patients who (after ECLND) are found to have occult RLN metastases have a 5-year survival rate of approximately 60%. In contrast, those patients found to be metastasis-free after ECLND have a 5-year survival rate of 80%.^{10, 15, 30, 32-36} Thus, by providing a means to histopathologically assess the status of the RLNs, the ECLND procedure allows a much more accurate assessment of prognosis than clinical nodal

staging alone.¹⁰ ECLND might therefore also be justified as an accurate melanoma patient staging procedure.²⁴

Despite the above-mentioned justification for ECLND, opponents of this procedure have adopted a 'watch and wait' approach after WLE, choosing to rather spare their patients from major surgery with its significant costs, potential risks and complications (e.g. acute wound problems, anaesthetic complications, chronic lymphoedema, nerve injury, etc.).³⁷ Their view is that RLN metastases are at best indicators but not governors of survival. Moreover, it is felt by some that removal of clinically-uninvolved RLNs could compromise the immune response against the tumour cells.^{38, 39} In support of this conservative approach, many studies have shown that patients with clinically-localised melanoma do not, as a group, seem to benefit from ECLND.⁴⁰⁻⁴²

Thus, ECLND has not generally been accepted as a therapeutic or nodal staging procedure in patients with clinically-localised melanoma. A more conservative means of identifying a subgroup of melanoma patients who are at high risk of harbouring occult RLN metastases has therefore been sought- these patients being more likely to benefit from ECLND. In this way those patients who were at low risk of having occult nodal metastases could be spared from undergoing unnecessary surgery.

1.2.2 Primary tumour characteristics

Much time, effort and expense have, in the past, been spent on identifying characteristics of the primary tumour and of the patient that would have prognostic value, and could therefore be used to accurately stage patients with clinically-localised melanomas. Although multiple variables (e.g. primary tumour site, patient age and gender) may add to the prognostic model,²⁴ only primary tumour thickness and ulceration have ultimately been shown to significantly influence prognosis in patients with clinically-negative RLNs (see Table 3 in Appendix A.22). Thus in the latest version of the American Joint Committee on Cancer staging system for cutaneous melanoma, tumour thickness and the presence/absence of ulceration are the clinical determinants used to accurately stage patients with no evidence of metastatic spread (see Tables 1 and 2 in Appendix A.22).¹⁰

However, it has also been shown that once the RLNs are involved by occult metastases, primary tumour thickness no longer accurately determines prognosis (see Table 3 in Appendix A22).¹⁰ It is therefore vital that an accurate assessment of RLN status be achieved; and since ECLND has not generally been accepted as a staging procedure for all patients with clinically-localised melanoma (see above), more emphasis has therefore been placed on identifying characteristics that could indicate a high risk for occult RLN metastases: It has been shown that the subgroup of patients with intermediate thickness melanomas (1-4 mm) are at high risk of having occult RLN metastases, but at relatively low risk of having distant metastases (i.e. locoregional disease). On the other hand, those patients with thick primaries (>4mm) are at high risk of harbouring both occult nodal and distant metastases.⁴³ In contrast, patients with thin primary tumours (<1mm) have a more than 98% cure rate with WLE alone, since the tumour cells are likely to still be confined to the epidermal compartment.⁴⁴

Based on the above findings, it would therefore seem more justifiable to perform a staging ECLND on just those patients with intermediate thickness melanomas, as the procedure is more likely to be of concurrent therapeutic benefit in this subgroup of melanoma patients. However, a large ten-year follow-up study assessing the benefit of ECLND in patients with intermediate thickness melanomas has shown no advantage of RLN removal compared to WLE alone. What the investigators did find is that only those patients with non-ulcerated limb melanomas of 1-2 mm thick seemed to benefit significantly from ECLND.¹⁸ This outcome might be explained by the finding that, in fact, only approximately 20% of patients with intermediate thickness primaries actually have occult nodal metastases. Therefore, up to 80% of melanoma patients selected (on the basis of having intermediate thickness melanomas) to undergo ECLND would gain no therapeutic benefit from this major operative procedure.^{45, 46} Thus primary tumour characteristics are not accurate enough to identify those patients who definitely have occult nodal metastases.

Whilst primary tumour characteristics were being evaluated as prognostic indicators, many investigators began to look at the possibility of analysing the blood compartment signs of primary tumour metastasis. The development of highly sensitive molecular techniques has contributed to the more accurate assessment of the blood for the presence of metastatic cells. Importantly, the blood compartment is very practicable to sample and since the vascular and lymphatic systems have numerous connections, the

evaluation of the blood might not only predict distant metastatic spread but could even predict early nodal involvement. For the past decade, a vast amount of research has focussed on the ability to better understand and detect early haematogenous melanoma spread.

1.2.3 Evaluation of peripheral blood to predict, or indicate the presence of early metastatic spread

The evaluation of peripheral blood for markers of metastatic spread can be achieved with minimal invasiveness, cost and inconvenience to the patient. Elevated levels of various serum markers, in particular lactate dehydrogenase (LDH), have been shown to be independent prognostic parameters in patients with metastatic melanoma.^{47, 48} However, a lack of specificity and sensitivity has limited their use in the detection of early metastatic spread.⁴⁹

In the last decade several tumour serum markers have also been assessed, with S100 protein and melanoma-inhibitory activity (MIA) showing the most promise. Elevated S100 serum levels have been shown to reflect disease recurrence in melanoma patients, and precedes other clinical/radiological evidence. However, it is this marker is not sensitive enough to serve as an indicator of microscopic disease.^{50, 51} Elevated MIA serum levels have been shown to correlate with advanced disease stage, and to have possible predictive value in patients with no clinical evidence of metastatic spread, although the latter is still inconclusive.^{49, 52}

Recently, Hanekom et al (2002) have shown that a reduction in the active fraction of plasma plasminogen activator inhibitor type 1 (PAI1), the main regulator of fibrinolytic activity in blood, could possibly indicate an increased risk for metastatic melanoma.⁵³ The detection of tumour-associated antigen TA-90 immune complexes⁵⁴ in the serum of surgically-cured melanoma patients has also recently been shown to accurately predict recurrence and survival,⁵⁵ and has been investigated in combination with positron emission tomography (PET) to detect occult metastases.⁵⁶ The sensitivity and specificity of this assay does not, however, approach that of histopathological RLN staging.⁵⁴ Moreover, independent research groups would need to validate the predictive value of this assay in order to confirm its apparent clinical value.

The use of RT-PCR technology for the detection of tumour cells in the blood was originally described in 1988 for chronic myelogenous leukemia.⁵⁷ With RT-PCR, tumour cell detection is no longer reliant on the need to characterise cancer-specific mutations,⁵⁸ but rather requires the identification of genes that are selectively transcribed in the tumour cells and not in the cells of the surrounding tissue.²⁵ The amplification of these mRNA targets would of course only be useful for the detection of tumour cells which have already metastasised from the tissue of origin.

RT-PCR was first applied to a solid tumour in 1991, for the detection of circulating melanoma cells (CMCs) in patients with advanced melanoma (having RLN or distant metastases).⁵⁹ Smith et al (1991) chose tyrosinase as their molecular marker firstly, because most melanocytic tumours express tyrosinase, and secondly, since the genomic organisation of tyrosinase was already known (allowing the design of intron-spanning PCR primers). Tyrosinase is one of the key enzymes in melanocytic differentiation and catalyses the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), and of DOPA to DOPAquinone, the first steps in melanin biosynthesis.⁶⁰ Its expression is restricted to melanocytes, melanocytic nevus cells, Schwann cells and most primary melanoma cells.⁶¹ Since the former three normal cell types are not normally present in the blood, it was hoped that the detection of tyrosinase transcripts in the blood of untreated melanoma patients would predict disease recurrence, or be indicative of residual subclinical disease (if detected after removal of existing disease).

A number of groups have now shown that tyrosinase RT-PCR can provide a very sensitive and specific assay for the detection of CMCs. *In vitro* studies show that it is possible to detect one tyrosinase-expressing melanoma cell in 1-10 ml of whole blood (equivalent to one melanoma cell in a background of 10^6 to 10^7 peripheral blood lymphocytes).^{59, 62, 63} Furthermore, investigators have shown that the specificity of the tyrosinase assay is almost 100%, as assessed by making use of blood from normal volunteers, or those with non-malignant disease.

The *in vivo* detection rate of the tyrosinase assay has generally been assessed by evaluating the blood of patients with advanced melanoma, since it is likely that CMCs will be present in the peripheral blood of patients with advanced disease. Although initially promising,^{59, 64} subsequent studies have shown the detection rate of the assay to be very inconsistent (ranging from 0%-100%)⁶⁵⁻⁷⁷ and to lack reproducibility.^{78, 79}

The clinical (predictive) value of detecting CMCs in the peripheral blood of melanoma patients with no clinical evidence of distant metastases has been determined by long-term follow-up studies of these patients. Results from these studies have provided conflicting data, with some studies supporting the detection of CMCs to determine prognosis,^{65, 66, 71, 74, 77, 79, 80} but others not recommending this approach.^{70, 77, 81-84} The inconsistent detection rate and predictive value of RT-PCR for the detection of CMCs may be attributed to various limitations of the molecular assay, as well as various biological factors, and these will be reviewed below.

In all the above-mentioned studies, tyrosinase was used as the sole marker for the molecular detection of CMCs. And since it has been found that melanoma cells may differ in their marker expression levels and profiles (with heterogeneity becoming increasingly evident in advancing stages of tumour progression⁸⁵ and as melanisation decreases^{74, 86, 87}), the use of a single-marker assay may have contributed to false-negative RT-PCR results.^{68, 73} Therefore, in order to improve the detection rate of CMCs in situations where melanoma cells do not express sufficient tyrosinase mRNA, the use of additional molecular markers has been investigated.⁷⁴ This might be particularly advantageous for the detection of hypomelanotic melanomas.⁸⁷ Candidate markers have included the mRNA's for a number of pigment cell-specific (PCS) markers, namely MART-1/Melan-A,^{73, 78, 87-92} Pmel-17/gp100,^{73, 87-89} Trp-1/gp75 and Trp-2/DOPA-chrome tautomerase.⁸⁷ These PCS markers are also often referred to as melanoma differentiation antigens (MDAs). Other candidates have included p97,^{73, 74, 92, 93} Muc-18,^{73, 74, 89, 92, 93} MIA⁹⁴ and the cancer/testis antigen MAGE-3.^{74, 93} The cancer/testis antigen (CTA) genes are expressed by most cancers,⁹⁵ but are silent in normal tissues other than testes and placenta.⁹⁶⁻⁹⁸ All these above markers are often referred to as 'melanoma-associated antigens' (MAAs) since most have originally been identified in melanomas. Other markers assessed include beta-human chorionic gonadotropin,⁹⁹ β 1-->4-N-Acetylgalactosaminyl-transferase¹⁰⁰ and ganglioside GM3.¹⁰¹ Several investigators have shown improvements in the *in vivo* detection rate of CMCs in patients with melanoma using a multi-marker assay, particularly when combining tyrosinase and MART-1.⁹⁰⁻⁹²

In addition to the problem of heterogenous marker expression, a lack of assay standardisation is also likely to have contributed to the inconsistent detection rate and predictive value of RT-PCR evaluation of the blood for melanoma cells.^{65, 86, 89, 102-104}

To address this the European Organisation for Research and Treatment of Cancer's Melanoma Cooperative Group (EORTC-MCG) has suggested standardised methods and standard quality control measures to avoid any PCR-based artefacts (false-positives), to improve assay efficiency (avoiding false-negatives) and to facilitate comparison of results from different laboratories.¹⁰⁴

It is highly likely, however, that the detection of CMCs in the blood of patients with advanced melanoma has been inconsistent primarily due to sampling errors: Due to the biological factor of intermittent shedding of tumour cells into the circulation, the time of blood sampling may not coincide with the shedding process.⁷⁶ Also, tumour cells persist only transiently in the peripheral blood,¹⁰⁵ being removed by shear forces within blood vessels and/or by active immune attack.¹⁰⁶ Furthermore, sampling of small blood volumes (10-20 ml) from the large blood pool (5 litres) seems to be statistically insufficient to obtain a positive RT-PCR test due to the low numbers of CMCs released into the vascular system at any given time. Sequential sampling (i.e. obtaining blood samples at different time points) does increase the detection rate but is likely to be non-feasible and too costly.^{81, 90} Thus sampling errors (more so than the assay itself) may significantly limit the detection rate of CMCs, and hence reduce the accuracy of peripheral blood evaluation to predict or indicate the presence of occult metastases.

Furthermore, since various biological factors are known to interfere with the successful establishment of metastases,^{72, 106-111} the detection of CMCs in untreated melanoma patients may not necessarily be predictive of recurrence. Thus, although venous sampling is a minimally-invasive, cheap and convenient means of screening patients, the molecular analysis of blood for metastatic melanoma cells has thus far, not been shown to be representative of disease stage or prognosis for melanoma patients.¹¹² Similar studies analysing bone marrow biopsies for metastatic melanoma cells via RT-PCR, have also not shown a correlation with prognosis.^{66, 82, 113} However, for other tumours (including breast, gastric, colorectal, small-cell lung and prostatic malignancies) the detection of tumour cells in bone marrow has correlated to a poor prognosis.¹¹¹

1.2.4 Lymphatic mapping, sentinel lymphadenectomy, and selective completion lymphadenectomy

The most promising recent development towards the improved staging of primary melanoma patients has been that of lymphatic mapping followed by sentinel lymphadenectomy (SL) (also known as sentinel node biopsy) and, where indicated, selective completion lymphadenectomy (SCLND). The lymphatic mapping and SL procedures involve nuclear medicine technology to guide the surgical removal of just 1-2 representative nodes in the RLN basin, thus providing a minimally invasive means of directly determining the status of the RLNs. This has been a revolutionary development because the histopathological status of the RLNs has been confirmed to be the most powerful predictor of survival in patients with melanoma:²⁴ Those melanoma patients found to have occult nodal involvement have been shown to have significantly reduced 5-year survival rates of approximately 50%, compared to approximately 80% for those patients whose nodes are negative by histopathological evaluation.^{10, 15, 30, 32-36} And since removal of the entire regional lymph node basin with an elective complete lymph node dissection (ECLND) (see section 1.2.1) is associated with significant morbidity and cost, a more conservative means of accurately sampling the RLNs of all those patients who are at risk for locoregional disease was needed. This is now possible by means of performing lymphatic mapping and SL, and this multidisciplinary approach has now become the standard of care for melanoma patients in many centres.¹¹⁴

How was this technology developed? The concept of the orderly lymphatic spread of cells from a primary tumour was first suggested by experiments in the 1900's.¹¹⁵ Thereafter, it was observed that the local injection and subsequent lymphatic uptake of a blue dye, radioactive colloid or radio-opaque contrast material allows the identification of the entire lymphatic drainage distribution, and particularly the first draining lymphatic basin of various malignancies (e.g. gastric, lung, testicular and cutaneous).¹¹⁶ Furthermore, every anatomic position has a very specific draining nodal basin, consistently identified by lymphatic mapping. Thus this procedure, performed pre-operatively by the nuclear medicine team, has overcome the problem of unpredictable and/or bi-directional lymphatic drainage, particularly for melanomas located on the trunk, head and neck.¹¹⁵ Merely following classic anatomic guidelines would have misdirected the ECLND, or missed another involved nodal basin, or in-transit node, in up to 75% of cases.^{24, 117, 118}

In the 1960's it was further noted that within a testicular nodal basin, if intra-operative lymphatic mapping was performed, the injected dye or colloid would always first drain to a specific lymph centre composed of usually 1-2 lymph nodes. In the 1970's the concept of additional intra-operative lymphatic mapping, with the identification and removal of these first-draining nodes was first developed for penile cancer. The hypothesis was that the tumour cells entering the lymphatic vessels would, like the dye or colloid, most likely first involve this same specific lymph centre. It was only in the 1990's that this procedure was re-awakened by Morton and colleagues and the term "sentinel" node (SN) was coined for the first-draining node in the RLN basin,¹¹⁵ with the term sentinel lymphadenectomy (SL) used to describe the procedure of surgically removing this node. More recently, the accuracy of correctly identifying the SN at surgery has been significantly improved by the introduction of a combination of the blue dye plus a radioactive agent, this being traced with a hand held gamma-detection probe.³⁷ The rate for successful identification of the SN in a patient now approaches 100% after a recommended learning curve of about 30 cases for each surgeon.^{45, 119} (see Figure 1.1).

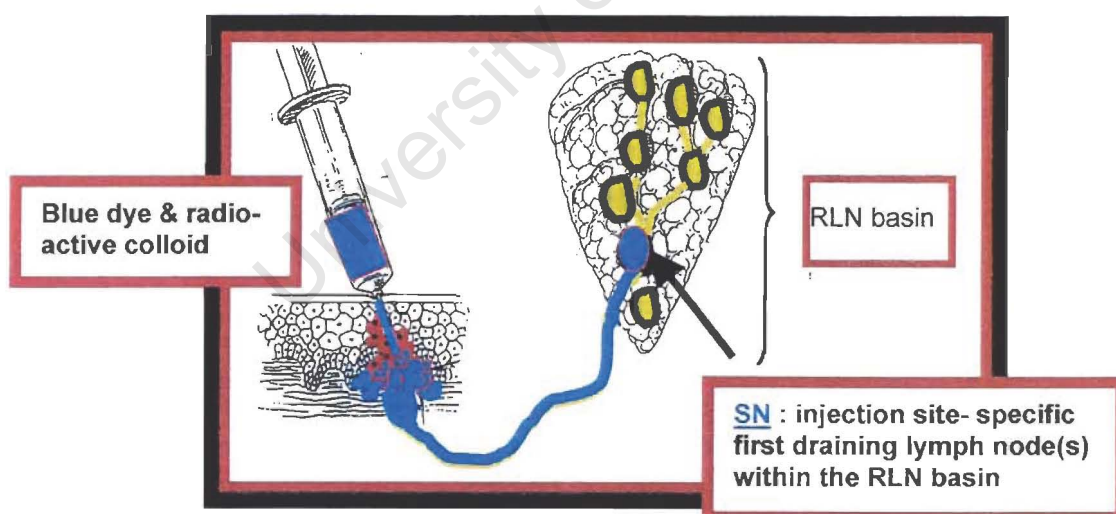


Figure 1.1 Schematic representation of the lymphatic drainage pattern of blue dye and radioactive colloid, after being injected at the site of the primary tumour. Diagram adapted from Cochran (1997).¹²⁰

Of note is that up to 30% of melanoma patients have more than one SN in either the same basin, in another drainage basin,^{45, 121-123} or occasionally as an in-transit lymph node¹²⁴ or aberrant node.¹²⁵ This emphasises the importance of performing pre-

operative lymphatic mapping, as well as a thorough re-examination of the surgical field once the identified SN has been removed, so as to detect any residual radioactivity that would indicate the presence of another SN.

The hypothesis that metastatic melanoma cells entering the lymphatic vessels would, like the dye and/or colloid, first drain to the SN was then confirmed: In fact, if the SN is found to be free of disease (by histopathological examination), then the remaining nodes in the RLN basin will also be uninvolved in 99% of cases.¹²² This orderly progression of melanoma metastases in the RLN basin has been confirmed by various other studies, with “skip” metastases found to occur in less than 4% of cases.^{46, 126-129} Thus only the SN needs to be surgically removed in order to determine whether or not metastatic spread has occurred to the RLNs. The important advantage of this procedure (compared to ECLND) is that it may be performed under local anaesthesia, is minimally-invasive, and is associated with minimal post-operative and long-term morbidity.^{130, 131}

Although SL is also gaining acceptance as a useful staging procedure for patients with gastric,¹³² thyroid,¹³³ vulvar¹³⁴ and other cancers,¹³⁵ the same “orderly progression” of metastases that is found to occur in the RLNs of melanoma patients is not always found to occur with other solid tumours. In particular, the incidence of “skip metastases” in breast cancer patients has been documented to be as high as 15%.^{136, 137} Therefore, the use of SL in breast cancer patients still raises some controversy.¹³⁸⁻¹⁴⁰ Of note is that this difference in metastatic progression may be attributed to the straightforward and reproducible pattern of lymphatic flow for a cutaneous malignancy,^{23, 46} as well as it being easier to inject the dye/radioactive colloid at the exact site of lymphatic tumour drainage.

At least for melanoma patients, the benefit of the advent of SL is that now all primary melanoma patients with a significant risk of locoregional disease (i.e. primary tumour thickness of 1–4 mm or primary tumour ulceration, microsatellitosis, angiolymphatic invasion and high mitotic rate) may have a full histopathological nodal staging without undergoing ECLND.^{141, 142} In fact, it has recently been reported that even patients with primary tumours more than 4 mm thick, but who are SN-negative, have a significantly better prognosis than SN-positive patients who have thinner primary tumours.¹⁴³ Therefore, SL has also been recommended for patients with thick melanomas.^{10, 142, 144}

Of those patients who are selected to undergo SL, 10-40% are found to harbour SN metastases.^{13, 115, 128-131, 142, 145-152} In approximately 30% of these cases, some of the other nodes in the RLN basin will also harbour metastases.^{45, 46, 121, 122, 142, 153-156} However, since it is not possible to know which SN-positive patient also harbours metastases in the remaining RLNs, the general approach is to perform a selective completion lymph node dissection in all patients whose SN is positive by histopathology.

In summary, the SL procedure now allows the RLNs of all melanoma patients at risk of having occult nodal metastases to be histopathologically staged without the need to perform an ECLND (see Tables 1 and 2 in Appendix A.22).¹⁰ Then only those patients with definite occult nodal involvement may be exposed to a selective completion lymph node dissection and adjuvant interferon in order to improve prognosis.²⁴ At the same time, those patients who have no SN metastases can be spared from undergoing unnecessary further surgery and/or adjuvant therapy. These patients can also be reassured of a good prognosis, with significant psychological benefit.¹²¹ Furthermore, SL identifies homogenous patient populations for entry onto clinical trials.

However, if analysis of this valuable SN tissue for the presence of melanoma cells is not accurate, this will negate the benefit of performing SL. Clinical trials assessing the outcome of melanoma patients undergoing SL have shown that, despite the improvements made in the histopathological evaluation of the SN, the detection of SN metastases is in fact sub-optimal due to inherent limitations of the technique. The following section reviews the literature with regards to developments in, and the current status of the histopathological evaluation of nodal tissue.

1.3 HISTOPATHOLOGICAL EVALUATION OF THE SENTINEL NODE

Historically, when ECLND was performed, a thorough examination of every node was not possible since up to 30 lymph nodes were typically submitted to the pathologist.¹⁵⁷ Performing a thorough examination of these nodes by serial sectioning would have been too labour-intensive and costly. Routine processing therefore had to be limited to making just one or two 4 µm sections from the centre of each node, which were then stained with haematoxylin and eosin (H&E).^{158, 159} Hence, less than one thousandth of the total nodal tissue submitted after ECLND was routinely examined,^{158, 160} resulting in a

significant risk of missing even larger metastases present in the nodal tissue that had not been sectioned. With the advent of SL, the surgeon can now provide the pathologist with just 1-2 SNs, which makes it more viable for the pathologist to more thoroughly examine the submitted tissue.

When SL was originally developed, it was also hoped that a rapid initial evaluation of the status of the SN via frozen section analysis could be performed in theatre. The reasoning was that if malignant cells were visualised in the frozen sections, selective completion lymph node dissection could still be performed in the same operating session as the SL. However, most pathologists now do not recommend the use of frozen sections to evaluate the SN for the following reasons: First, up to 50% of the nodal tissue may be lost during the preparation of frozen sections. Freeze-artefacts may also be introduced, thus compromising the subsequent H&E examination that is performed if the frozen sections are negative.^{121, 161, 162} Secondly, the identification of small numbers of melanoma cells is significantly more accurate in well-fixed and stained sections.¹³ Thus, despite the advantages of frozen section analysis of the SN, it is nevertheless recommended that evaluation of the SN be performed on formalin-fixed wax-embedded sections.¹⁴⁹

A more thorough examination of the SN is now possible by making serial sections of the fixed tissue. One respected recommendation is that 20 serial sections be made, preferably from the central portion of the node.¹³ Another recommendation is that sections at three different levels be made (i.e. step sections at approximately 50-100 micron intervals) from each half of an SN.^{159, 161} Examination of these deeper levels of the node has been shown to increase the detection of melanoma cells within the node. However these recommended SN sectioning methods, which are quite labour intensive and therefore not performed by all investigators, allow the examination of only 1-5% of the SN at best.¹³⁰ Thus, although these methods will easily enable the detection of larger SN metastases, microscopic SN metastases (micro-metastases) that are composed of isolated tumour cells, or clusters thereof, may still easily be missed.¹¹¹ By definition, these clusters may vary in size, being anything less than 0.5¹⁶³ or 2 mm.¹⁶⁴

In addition, single cells and small clusters may still go undetected using routine H&E staining, even if these cells are present in the SN section examined.^{13, 152} This is because H&E staining has a limited sensitivity of allowing the detection of one tumour

cell in a background of 10^4 to 10^5 cells.^{24, 165} This may be compared to immunohistochemical (IHC) staining which allows the detection of one malignant cell in a background of 10^5 to 10^6 cells.^{146, 166} It is for this reason that additional IHC staining of SN serial sections is recommended if no metastatic cells are detected with conventional H&E.¹³

As discussed above, the use of IHC staining of the SN sections improves the sensitivity of histopathological evaluation. However, the limitation of these IHC markers is that some metastases may still be missed because there are currently no commercially available antibodies that have a 100% detection rate. Furthermore, non-specificity of some of the IHC markers within a nodal section may lead to false-positive results.¹¹¹

The antibody to the cytoplasmic S-100 proteins (polyclonal anti-S100) and/or the melanoma marker Pmel-17/gp100 (monoclonal HMB45), are conventionally used to detect melanoma nodal metastases.^{13, 149, 154, 159, 161} The S100 antibody has a high detection rate for melanoma, but has the disadvantage of poor specificity as it stains many cell types other than melanoma cells (e.g. nodal interdigitating dendritic cells, certain sinus macrophages, Schwann cells of node-associated nerves and nodal neval cells). This results in high confounding background staining, with the potential to miss individual tumour cells.¹³⁰ In contrast, Pmel-17/gp100 (a melanosomal matrix glycoprotein) has a rather poor detection rate since its expression is frequently lost during tumour progression.¹⁶⁷⁻¹⁶⁹ Up to 30% of melanoma nodal metastases do not stain with HMB45,^{130, 154, 170, 171} and heterogeneity of expression exists even within a melanoma mass. However, the advantage of this marker is that it is very specific for cells of the melanocytic-lineage.¹⁷²⁻¹⁷⁴

Many other melanoma-associated antigens (MAA's) have been assessed for their potential as IHC markers for the detection of melanoma nodal micro-metastases.^{175, 176} NK1C3 (a monoclonal antibody to an unidentified melanoma-associated antigen) is more sensitive than HMB45, but like the S100 antibody lacks specificity and moreover, is very costly.¹³⁰ The A103 antibody to MART-1/Melan-A (Melanoma Antigen Recognised by T-cells) has recently been found to be a very useful additional antibody for the detection of melanoma metastases. It has a detection rate similar to the S100 antibody, but with no background staining of non-melanocytic cells within the node.^{130, 171} Although MART-1/Melan-A is claimed to be expressed in 80-100% of primary

melanomas,¹⁷⁴ expression levels are lost in a high percentage of metastatic melanoma lesions.^{13, 154, 167} Some studies have however found MART-1 to have a high detection rate for metastatic melanoma.^{130, 177} MAGE-3, a member of the 'melanoma antigen-encoding gene' A (MAGE-A) family of cancer-testis antigens (CTAs), is another potentially useful IHC marker since it does not stain normal nodal cells,¹⁷⁸ and is expressed in approximately 60% of metastatic melanomas.¹⁷⁶ An antibody to the tyrosinase protein, T311, has recently become available. Like MART-1/Melan-A, tyrosinase is expressed by most primary melanomas,^{174, 179} but heterogeneity of expression is found in melanoma metastases.¹⁶⁹ Therefore, although many IHC markers are available, and a combination of these is likely to improve the detection rate for melanoma nodal micro-metastases, a 100% detection rate is still unlikely due to heterogeneous antigen expression. Moreover, melanoma cells that are present as small clusters, or as single cells, may still be undetectable due to the confounding background staining of certain normal nodal cells with some IHC markers.

In addition, the positive staining of nodal background cells, in particular nodal neval clusters (found in some melanoma-draining SNs-see section 1.4.2.3), may be mistaken for melanoma micro-metastases. However these benign cells may be differentiated from melanoma cells by re-examination of the previously negative, adjacent H&E section, since the benign cells show no atypia or mitoses.¹⁴⁷ Moreover, it has been shown that nodal neval cells do not stain with MAGE-3,^{130, 149, 176, 180} and have low or no staining with HMB45.^{128, 130, 149} A further distinguishing feature between the nodal nevi and melanoma metastases is that the former are found only within the capsule or trabeculae of the node, and never in the subcapsular or medullary spaces, where the tumour cells are usually found.¹⁸⁰ Therefore, as long as a positive IHC is confirmed by H&E of the adjacent tissue section, non-specific IHC staining of nodal neval cells should not produce any false-positives.

Lastly, the accuracy of the histopathological examination of the lymph node is also affected by the manner in which the tissue is excised, preserved, and the timeliness with which it is transferred to the pathology laboratory.¹³ The tissue should therefore be placed in formalin immediately after excision. In the laboratory, after meticulous gross examination of the SN, the node is bisected through the longest circumference and each half paraffin-embedded for sectioning.

In conclusion, the histopathological evaluation of the SN for the detection of melanoma micro-metastases is significantly limited because, at best, only 1-5% of the node can be examined by serial sectioning. Furthermore, although the use of 1-2 IHC markers will improve the sensitivity of histopathological SN evaluation, the heterogeneity of IHC marker expression in metastatic melanomas is likely to result in an unacceptably high false-negative rate. In addition, the use of serial sectioning and IHC staining is extremely labour intensive, costly and therefore not routinely done in many institutes. Thus, an alternative, more sensitive and cost-effective means of SN evaluation continues to be sought. Molecular evaluation of the SN is an attractive alternative or complimentary technique as it offers a variety of advantages over IHC, and these will be reviewed next.

1.4 THE USE OF RT-PCR FOR THE DETECTION OF MELANOMA NODAL MICRO-METASTASES

An RT-PCR technique has previously been developed for the detection of circulating melanoma cells (see section 1.2.3). This technique could easily be adapted for the detection of melanoma cells in nodal tissue by modifying the RNA extraction protocol to allow for the homogenisation the SN tissue.¹⁵⁸ The molecular evaluation of the SN has several advantages over standard histopathological nodal examination: Most importantly, RT-PCR would allow for the examination of the entire lymph node and would therefore overcome the risk of sampling error that might occur with histopathological examination of the SN.¹⁵⁸ In addition, RT-PCR evaluation of the entire SN is likely to be significantly less labour-intensive and costly than evaluating just 1-5% of the tissue submitted for IHC examination.

Further advantages of the molecular evaluation of the SN are that sensitivities achieved with RT-PCR are reported as being 1-2 orders of magnitude higher than those achieved with histopathology,¹⁴⁶ RT-PCR allowing the detection of one melanoma cell in 10^6 to 10^7 background cells.^{112, 146, 148} RT-PCR might therefore detect additional very small/early tumour burdens ('submicroscopic' metastases) that are missed by recommended histopathology. Moreover, RT-PCR is amenable to the cost-effective use of multiple markers, which is likely to improve the detection rate of the assay and may also be of prognostic value. Lastly, RT-PCR is relatively rapid and more easily standardised and automated,¹¹¹ particularly with the advent of real-time PCR. Therefore,

based on all these significant advantages afforded by the molecular evaluation of nodal tissue, it would seem ideal to use RT-PCR as a standard of SN evaluation. Besides being investigated for its use in melanoma patients, RT-PCR is also being investigated as a tool for the identification of nodal micro-metastases in various other solid tumours.^{20-22, 181-183}

However, the very fact that the technique is so sensitive is unfortunately also the reason for the associated risk of producing false-negatives and particularly false-positives. The following two subsections will review the potential causes of technical false-negative and false-positive RT-PCR results.

1.4.1 Potential causes of a technical false-negative result

For purposes of this discussion, a technical false-negative is defined as a negative RT-PCR result where melanoma cells were in fact present in the tissue being evaluated. The above-described advantages of RT-PCR over the IHC evaluation of the SN would indicate that, at least in theory, the molecular approach would carry a lower risk of technical false-negatives. However, when there are very low numbers of melanoma cells in the SN (as with micro-metastases), and therefore a low concentration of mRNA template molecules in the RNA extract, the risk of producing a technical false-negative RT-PCR result is nevertheless still possible. The following three factors are most likely to contribute to the majority of technical false-negative results:

1.4.1.1 RNA degradation. RNases, which degrade RNA, are released during cell lysis but may also be introduced from external sources (e.g. hands or lab ware). RNA degradation may occur at any stage during the molecular protocol until cDNA is synthesised (particularly prior to RNA extraction of the tissue) and may significantly reduce the sensitivity of RT-PCR analysis.^{89, 104} Furthermore, if specific mRNA target molecules are scarce, then even a minor variation in the amount of RNA degradation occurring may lead to a sporadic false-negative result.

1.4.1.2 RT-PCR inefficiency. RT-PCR efficiency may be compromised by user technique, design of the nodal homogenisation protocol, RT-PCR parameters (e.g. RT extension time, annealing temperature and cycle number), primer design, reagent quality (in particular the reverse transcriptase and DNA polymerase enzymes) and PCR machine used. These aspects of the protocol should therefore all be addressed in order

to achieve maximum RT-PCR efficiency and hence sensitivity. It has furthermore been shown that the RT-step is the main source of variability in RT-PCR efficiency, with the RT enzyme being sensitive to salts, alcohols, phenol or protein contaminants that may remain from the RNA extraction step.^{111, 184} Therefore variations in the efficiency of the RNA extraction may reduce the RT-efficiency, and may result in sporadic false-negatives when mRNA target molecules are scarce.

1.4.1.3 Tumour heterogeneity.

Since heterogeneity of marker expression becomes increasingly evident in advancing stages of tumour progression,⁸⁵ both IHC and RT-PCR may lead to false-negative results when an SN is being evaluated for micro-metastases, particularly if only one marker is used. Since it has been shown that multi-marker analysis of the blood for CMCs improves the detection rate compared to single-marker analysis with tyrosinase (see section 1.2.3), similar approaches have been pursued for the detection of melanoma cells in other tissue targets. It is now evident that a multi-marker approach increases the detection rate of an RT-PCR assay when evaluating macroscopic melanoma metastases.^{87, 95, 185, 186} The extent of marker heterogeneity in microscopic nodal metastases has not been extensively evaluated,¹²⁸ however it is likely that such a multi-marker approach will also be advantageous.

1.4.2 Potential causes of a technical false-positive result

Of more concern than the risk of false-negatives is the risk of producing technical false-positives when using RT-PCR to evaluate the SN. For purposes of this discussion, a technical false-positive is defined as a positive RT-PCR result in the absence of melanoma cells in the tissue being evaluated. Although some studies have found tyrosinase mRNA expression to be specific for melanoma-involved tissues and cells of the melanocytic lineage,^{75, 87, 126, 128, 151, 152, 158, 187} others have reported the detection of tyrosinase transcripts in some normal tissues, as well as tissues involved by benign disorders and also some non-melanoma tumour-involved tissues (including tumour-involved lymph nodes).^{61, 112, 148, 188} Likewise, Pmel-17 mRNA transcripts have been detected in normal tissues including lymph nodes,¹⁷³ although others have found Pmel-17 expression to be absent in normal tissues and non-melanoma tumour-involved nodes.⁸⁷ Unfortunately, unlike with histopathology where the cellular origin of the specific IHC target antigen can be confirmed by H&E, a positive RT-PCR result cannot be microscopically confirmed. Thus the detection, by some investigators, of some pigment

cell-specific (PCS) transcripts in the lymph nodes of non-melanoma patients is currently the greatest obstacle to ongoing progress in this field, with many investigators feeling that the use of RT-PCR for the detection of melanoma cells in nodal tissue has limited diagnostic value. The following three sub-sections review the most likely causes of technical false-positive RT-PCR results.

1.4.2.1 Carry-over/amplicon contamination. Sporadic false-positives resulting from amplicon contamination are more often associated with 2-stage PCR reactions (e.g. nested-PCR). During the preparation of the PCR product dilutions that are used in the second round of PCR, there is an increased risk of releasing aerosolised amplicons, and especially of contaminating the pipette used for subsequent samples. This risk may be reduced by making use of aerosol-resistant tips. An alternative approach has been to develop a 1-stage PCR protocol, as described by Pittmann and co-workers.⁷⁶ Their 50-cycle PCR protocol was as sensitive as their previously described nested-PCR method.⁵⁹ Since then, others have adopted a similar approach.^{94, 189}

1.4.2.2 Amplification of residual DNA in the RNA extract. Most RNA extracts contain a certain amount of residual DNA¹⁸² that may be amplified if the PCR primers have not been carefully designed. However, even if the primers appear to be cDNA-specific it is still recommended that a cDNA-negative control be performed for each primer set when initially optimising the PCR reactions. By convention, a cDNA-negative control is performed by either omitting the reverse transcriptase from the RT-step, or by omitting the RT-step and performing PCR directly on RNA. Alternatively, this control can also be performed on purified DNA. This cDNA-negative control will also exclude the existence of a pseudogene for that particular sequence of the gene: If the cDNA-negative control is unexpectedly found to yield a product of the identical size as that expected from the cDNA template, and the water-negative control remains negative (which excludes amplicon contamination), then this is most likely indicative of the existence of a pseudogene for that particular gene.¹⁹⁰ These pseudogenes tend to lack the normal intronic sequences and have presumably arisen from the integration of reverse-transcribed mRNA into the genome.^{182, 183} If present, these sequences are most often highly homologous to the target cDNA and may therefore result in technical false-positives. Treatment of the RNA extract with DNase, prior to performing RT-PCR, will allow confirmation of the presence of a pseudogene. If this were the case, then it would be advisable to design a new set of primers that will not co-amplify the pseudogene. If

not possible, alternative pseudogene-less targets should be considered, or else the RNA extract should be routinely subjected to DNase-treatment.¹⁹¹

1.4.2.3. Detection of unwanted transcripts. When choosing a molecular marker for the specific detection of a certain cell type in a background of other cells, it is obviously very important to select markers that are not expressed by the background cells. The PCS markers and cancer/testis antigens (CTAs) used in previous studies for the detection of melanoma cells in nodal tissue have been chosen because of their reported specificity for pigment and malignant cells respectively, their expression being absent in the majority of normal nodal cells. However, it has recently been highlighted that there are small populations of cells in lymph nodes (i.e. nodal Schwann and neval cells), which also express the PCS markers.

(i) Schwann cells are histologically detectable in all lymph nodes,¹²⁸ and have been shown to express tyrosinase.^{95, 192} In SNs, these cells stain positive for S100 but are negative for HMB45 (i.e. the Pmel-17 antibody).¹²⁸ Although very few Schwann cells are present in lymph nodes, the extreme sensitivity of RT-PCR may lead to the detectable amplification of Schwann cell-specific PCS transcripts in normal control nodes.

(ii) Nodal melanocytes/neval cells are reported to be present in approximately 4% of all melanoma-draining SNs,^{130, 149, 154, 180} and in approximately 4-22% of melanoma patients.^{128, 130} Only approximately 1% of non-SNs within a melanoma-draining RLN basin contain nodal nevi.^{130, 131, 146, 149, 180} The nodal incidence of nevi in non-melanoma tumour-draining nodes is significantly lower (approximately 0.04%)¹⁴⁹ than for melanoma-draining SNs. No nevi have been detected in non-skin draining nodes,^{13, 180, 193} and the incidence of nodal nevi in normal skin-draining nodes has, to the best knowledge of the investigator, not been reported. Of interest is the high incidence of benign breast epithelial cells in the breast-draining lymph nodes of patients with malignant, as well as benign, breast disease. Likewise other epithelial cells have been identified in nodes that drain other respective epithelial tissues (whether this tissue is involved with benign or malignant disease).¹⁹⁴

Two hypotheses have been put forward to explain the existence of nodal nevi: First, aberrant neural crest cell migration during embryogenesis, and secondly, endolymphatic transport of melanocytes from cutaneous nevi to nodes (the "benign metastasis" theory).

At present, evidence to prove either hypothesis correct remains inconclusive, although the second hypothesis better explains the absence of nodal nevi in non-skin draining nodes. Cutaneous nevi might also become displaced by the growth of an adjacent melanoma and thus both could enter the same draining lymphatic vessels. This might explain the finding that nodal nevi are highly associated with the presence of a cutaneous melanoma.¹⁸⁰

Since nodal neval cells have similar morphology and expression profiles as dermal melanocytes,^{61, 180} they are also very likely to express certain PCS marker mRNA transcripts. This would not be problematic if nodal nevi were present only in melanoma-draining SNs that also contained metastatic melanoma cells. Unfortunately, in fact the incidence of nodal nevi in melanoma-draining SNs that are micro-metastasis-free by histopathology has been reported as being 8.5%. Thus, this means that a certain number of histopathologically SN-negative patients may be incorrectly evaluated as being SN-positive by RT-PCR analysis. It may be seen as reassuring, however, that Bostick et al (1999) have shown no detectable tyrosinase, MART-1 or MAGE-3 mRNA transcripts in melanoma-draining SNs that were histologically free of metastases but found to contain nodal nevi.¹²⁸

(iii) Illegitimate Transcripts: Genes can be divided into housekeeping genes that are expressed in essentially all cells and encode common structural proteins or ubiquitous enzymes, and tissue-specific genes where expression is limited to one or more specific cell types. However it has been shown that the latter genes may also be transcribed at a very low level in any cell type, this phenomenon being termed illegitimate or ectopic transcription.^{195, 196} It is unlikely that these very 'low abundance' transcripts (estimated to be one mRNA molecule/100-1000 cells)¹⁹⁷ have any physiological function. When using RT-PCR it is important to remember that these illegitimate transcripts may become detectable when the number of PCR cycles used is high (usually above 40 PCR cycles).¹⁹⁶⁻¹⁹⁹

Despite the above-reviewed potential causes of technical false-positive and false-negative RT-PCR results, many investigators have nevertheless maintained that RT-PCR evaluation has the potential to significantly improve the detection of occult melanoma metastases in the SN. In order to investigate whether an optimised RT-PCR assay is indeed superior to histopathology for SN evaluation, it is obviously necessary to

carry out research where the two techniques and their clinical relevance are compared. This requirement, however, brings to light a very complex technical and ethical problem that makes the above comparative studies very challenging. The problem is as follows: The pathological examination of the SN is currently the accepted standard of SN evaluation, and determines the prognosis and further management of the patient. Since at this stage the results of the molecular evaluation of the SN are purely for research purposes, if the RT-PCR result differs from the pathological report, it will not currently affect patient management. For this reason the amount of each SN that can be used for RT-PCR is limited. Certainly, until the validity of SN evaluation using RT-PCR is confirmed, patient care must not be compromised by the loss of large SN portions to research.^{13, 200} Therefore some investigators report the use of only small fractions of the SN for evaluation of their RT-PCR protocol.^{131, 152, 187} Other investigators have made use of nodal cryostat sections for RT-PCR, with the alternating sections being used for histopathological evaluation in order to circumvent this risk. However, since it has been shown that frozen sectioning of the nodal tissue can disrupt the cellular morphology by up to 50%,^{161, 162} it is unlikely that most pathologists will agree to perform frozen sections on valuable SN tissue.

Most investigators comparing molecular and histopathological evaluation of the SN now tend to use half of the SN for each technique.^{126, 146-148, 158, 201-203} This gives investigators a chance to accurately compare RT-PCR assessment with histopathological assessment of the other half of the SN. However, many pathologists have raised the concern that bisecting the SN may compromise the histopathological examination of the SN, since metastases may be limited to the half used for RT-PCR analysis. However, it has been shown that melanoma metastases are very often identified as scattered cells or small clusters throughout the subcapsular space and trabeculae of the node,^{13, 152} and preferentially at the hilum.^{36, 130} Thus careful longitudinal bisection of the SN through the hilum, with the one half of the SN being used for RT-PCR, is unlikely to compromise the histopathological detection of micro-metastases (although bisection of the node is not always precise when it is very small). A possible solution for the above-mentioned concern might, however, be to use *RNA/ater* (Ambion, Texas, USA) instead of conventional formalin fixation. The advantage of *RNA/ater* is that not only does it appear to preserve the histological and IHC parameters of tissues, but it also effectively preserves tissue mRNA.²⁰⁴ Thus RT-PCR evaluation

could be performed on intervening nodal tissue sections that were not used by the pathologist.

Ultimately, what needs to be compared is the accuracy of RT-PCR to predict recurrences, versus the accuracy of IHC to do the same. The outcomes of these studies have shown that RT-PCR is able to detect additional, clinically-significant SN metastases that are missed by routine histopathology. The final section reviews the significant improvement in patient prognosis and management that has been possible by the development of SL and histopathological evaluation of the SN, and compares the clinical (predictive) value of IHC to that of RT-PCR evaluation of the SN.

1.5 CLINICAL SIGNIFICANCE OF THE STATUS OF THE SENTINEL NODE (SN) AND THE ADVANTAGES OF RT-PCR EVALUATION OF THE SN

It has now clearly been shown that the status of the SN impacts significantly on prognosis: Patients who are SN-positive by histopathological evaluation have been shown to have a significantly poorer prognosis than those found to be metastasis-free (histologically), even though the former undergo a potentially curative selective complete lymph node dissection.^{127, 128, 145, 147, 205} This difference in the 5-year survival rate is particularly significant in patients with primary melanomas of 1-4 mm (with or without ulceration), and also those patients with melanomas more than 4 mm thick without ulceration: An average 5-year survival rate of 82% for SN-negative patients versus 50% for SN-positive patients.¹⁰ The histological status of the SN has thus been shown to be the most important prognostic factor in primary melanoma patients.^{3, 127, 145, 203}

Not only does SN histopathological evaluation allow a more accurate prognosis, but it also identifies patients who might benefit from adjuvant therapy and ECLND. In 1995, Interferon alfa-2b (IFN α -2b) became the only Food & Drug Administration (FDA)-approved adjuvant systemic therapy for the treatment of metastatic melanoma.¹⁶ The greatest benefit of IFN α -2b therapy has been achieved in patients with metastatic RLN involvement, including patients with sub-clinical RLN metastases. Moreover, patients with confirmed occult nodal metastases may represent the most appropriate group to study potentially useful immunotherapeutic strategies, especially since large tumour burdens have been shown to inhibit anti-tumour immunity.²⁰⁶

Thus with the advantages of the histopathological evaluation of the SN evaluation now being so clear, the World Health Organisation (WHO) has recently issued a consensus statement recommending that SL should become the new standard of care for primary melanoma patients.¹¹⁴ The American Joint Committee on Cancer (AJCC) has also recommended that all primary melanoma patients who have no clinical evidence of RLN spread, and who have primary tumours greater than 1 mm thick or ulcerated tumours less than 1 mm thick, should undergo histopathological nodal staging prior to entering clinical trials.¹⁰ However, these selection criteria are still subjects of debate.¹⁴² At institutes where the interdisciplinary collaborations are not yet in place to allow for SL, it is recommended that ECLND be offered to all patients with melanomas more than 1 mm thick (Guidelines from Intergroup Melanoma Surgical Trial).²⁴

The results of the histopathological evaluation of the RLNs have now been incorporated into the new, revised (2002 version) Melanoma Staging System. Patients with SN-metastases that are detectable by pathologic examination only are now upstaged from clinical Stage I or II to pathological Stage III because of the significant implication of occult nodal involvement on prognosis (see Table 1 in Appendix A.22).¹⁰ This revised staging system has been approved by both the AJCC and the International Union Against Cancer (UICC) tumour-node-metastasis (TNM) committees, and will become official with publication of the AJCC *Cancer Staging Manual of 2002*.

Despite the significant prognostic value of the histopathological status of the SN it has been shown that only approximately 31-67% of SN-positive patients actually develop clinically-detectable recurrences.^{128, 146, 203} This means that there is an overall false-positive SN rate of approximately 30-70%. The histopathological evaluation of the SN is unlikely to contribute to this false-positive SN rate if H&E confirmation of the presence of melanoma cells is routinely performed. Rather, various biological factors are likely to contribute to the lack of recurrences in some patients where the SN is shown by histopathology to harbour melanoma cells: First, isolated tumour cells in lymph nodes may be there purely as a result of tumour cell shedding caused by angiogenesis within, and vascular invasion of the primary tumour.²⁰⁷ These cells might not have any metastatic potential, and may never evolve to become macroscopic metastases.¹¹¹ Therefore the detection of melanoma cells in the SN is not always synonymous with active metastatic disease. Secondly, since the RLNs are known to act as temporary filters for metastatic cells, it might be that in some patients metastatic spread has not yet

occurred past the RLN basin. Spread might not even have occurred past the SN, since in more than 70% of SN-positive patients the other RLNs are free of metastasis by IHC evaluation. Therefore, some SN-positive patients may be surgically cured by selective completion lymph node dissection, and even SL alone. Thus the value of selective completion lymph node dissection and/or adjuvant therapy following every positive SN finding would also have to be questioned.^{142, 150} Lastly, and very importantly, even if micro-metastatic spread has already occurred past the RLN basin in SN-positive patients, clinical recurrences might not develop during the usual follow-up period (10 years or less). This might be explained by the finding that the cells of micro-metastases appear to be in a “dormant” state (as determined by their proliferative profiles) compared to those cells making up macroscopic metastases.²⁰⁸ Clinically-detectable metastases might therefore not emerge from these micro-metastases for many years. Much longer follow-up (more than 10-15 years) of these patients might reveal that the false-positive SN rate is actually much lower than currently thought.^{111, 209, 210} Unfortunately, no pathologic features of the primary tumour or SN have yet been identified that will accurately predict which SN-positive patients will also have positive non-SNs or distant microscopic spread. Multi-marker profiling and quantitation of tumour burden in SNs may allow the identification of SN-positive patients who are likely to benefit from further therapy. At this stage, however, selective completion lymph node dissection and interferon therapy are recommended for all patients after a positive SN finding with histology,^{45, 131, 142, 150} although not all agree with this approach.²¹¹

Despite the good prognosis for patients with histologically-negative SNs (as a group), it has nevertheless been shown that up to 25% of patients who are SN-negative by histopathology, still develop recurrences.^{13, 14, 24, 115, 130, 145-148, 155, 159, 205, 212} As with the false-positive SN cases, some of the false-negative SN cases may arise due to biological factors: First, a proportion of these SN-negative patients develop systemic recurrences, pointing to the possibility that some patients have pure haematogenous spread directly from the primary tumour.¹⁴ Secondly, if nodal recurrences occur after, or simultaneously with local or in-transit recurrences, it could point to residual local disease, or a missed in-transit SN as the cause of a false-negative result (even though the histopathological result was a ‘true-negative’). However, the most common site of first recurrence in the SN-negative patients is the RLN.^{14, 145, 205} In a minority of cases this may reflect surgical misidentification of the true SN, or a missed second SN in the same or different nodal basin, or the biological occurrence of skip metastases (see section

1.2.4). However, the majority of these false-negative SN cases seem to arise due to the sub-optimal detection of SN micro-metastases (even if additional IHC and serial sections have been used) These histopathological false-negatives most likely arise because, as mentioned previously, only 1-5% of the SN can be examined (at best).^{24, 115, 146, 147, 205}

Here lies the significant advantage of additional molecular evaluation of the SN: Studies have shown that RT-PCR evaluation of SN tissue is significantly more sensitive than histopathological evaluation. SNs that are positive by histology are almost always also positive for tyrosinase RT-PCR.^{128, 131, 146-148, 151, 152, 158, 187, 202, 203} In addition, the latter seems to allow the detection of melanoma metastases in the SN that would otherwise be missed by histopathology. These studies have found that RT-PCR would result in the upstaging of approximately 20% (and in some studies even up to 60%) of primary melanoma patients who have been found to be tumour-free by histopathological evaluation of their SN(s).^{75, 126, 128, 131, 146-148, 151, 187, 203, 213}

The clinical significance of being able to more sensitively detect occult metastases has been confirmed by the comparison of recurrence rates in patients who have had their SNs evaluated by both techniques: Those patients who are SN-negative by both RT-PCR and IHC, have a significantly lower recurrence rate (0-6%) than those patients shown to be SN-negative by IHC examination alone (up to 25%-see above)(see Figure 1.2). Those patients who are upstaged by molecular evaluation (i.e. RT-PCR-positive but IHC-negative) have an intermediate recurrence rate of 13-25%, this being compared to a recurrence rate of approximately 31-67% for those patients who are SN-positive by both techniques.^{128, 146, 203}

SN status:	IHC-positive RT-PCR-positive	IHC-negative RT-PCR-positive (molecularly-upstaged)	IHC-negative RT-PCR-negative
Recurrence rate:	31-67%	13-25%	0-6%

Figure 1.2. Summary of the intermediate risk of recurrence in melanoma patients who are upstaged by RT-PCR evaluation of their SN

Importantly, what the above results indicate is that technical false-negatives are less likely to occur with RT-PCR than with histopathological evaluation of the SN. Furthermore, although the recurrence rate in the patients upstaged by RT-PCR is lower than in those patients who are positive by both techniques, the recurrence rate in the upstaged patients is still significantly higher than for patients who are SN-negative by both techniques. This confirms that nodal melanoma metastases detectable by RT-PCR analysis alone (i.e. these have been missed by histopathology) are clinically-significant, since they develop into clinically-detectable recurrences. Importantly, it implies that metastatic spread has already progressed beyond the SN to the remaining RLNs, and even to distant sites, in at least in a proportion of these molecularly-upstaged patients.²¹⁴ Therefore these patients (like the IHC-positive patients) are also likely to benefit from early aggressive surgery and adjuvant therapies. The Sunbelt Melanoma Trial is currently investigating this by randomising patients, who are IHC-negative but RT-PCR-positive, to selective completion lymph node dissection with or without high-dose IFN α -2b therapy, versus observation alone.^{115, 215}

It cannot, however, be overlooked that whilst RT-PCR evaluation of the SN is more sensitive for the identification of clinically-significant nodal melanoma metastases, there is also a concurrent increase in the false-positive rate when RT-PCR analysis is used (i.e. a lower percentage of patients who are RT-PCR positive will develop recurrences than patients who are IHC-positive). This could partly be explained by the likelihood that the micro-metastases identified only by RT-PCR are very small, and therefore less likely to be clinically significant, or to have spread past the SN. However, the molecular detection of specific PCS transcripts in control tissues, as reported by some investigators (see section 1.4.2) suggests that particular attention will have to be placed on limiting the risk of RT-PCR-induced (technical) false-positives in order to reduce the overall false-positive SN rate.

In conclusion, the early detection of occult metastases remains a huge challenge for those involved in the care of patients with melanoma as well as other malignancies. The improved detection of nodal micro-metastases holds the key to many basic questions on the initiation of metastasis and would open the doorway for the testing, and ultimately, the application of tumour immunotherapy. Although RT-PCR provides a potentially very useful and cost-effective technique for the detection of SN micro-metastases, its current limitation is that it over-estimates the number of patients who have clinically-significant

melanoma metastases. Therefore in order to make this molecular tool more acceptable for routine clinical use, the following steps need to be taken to minimise technical false-positives: First, the specificity of every optimised assay must be assessed on adequate controls prior to evaluating SNs. Secondly, it is also vital to ensure that the presence of PCS-expressing nodal neval cells will not result in a technical false-positive RT-PCR result. Thirdly, routine controls must be performed with every patient sample to exclude sporadic false-positives. Finally, it seems that it will be necessary to reduce the sensitivity of the RT-PCR assay for melanoma cells in nodal tissue in order to detect just those metastases that are clinically-significant.

1.6 RATIONALE

The worldwide incidence of cutaneous melanoma has been increasing at an alarming pace, with South Africa and Australia, having the highest incidence rates.^{4, 216} Sentinel node (SN) biopsy has been recommended by the World Health Organisation as the new standard of care for the management of melanoma patients with a primary tumour more than 1 mm thick, and who have no clinical evidence of nodal involvement.²⁸ As explained above, this allows for the accurate (sub-clinical) staging of the RLNs in primary melanoma patients, and this has become increasingly important because effective adjuvant therapy is now available.¹²⁸

A gamma-detection probe for the identification of the SN has recently been donated by a private company, jointly to Groote Schuur and Tygerberg Hospitals. The SN biopsy procedure has been validated at Groote Schuur Hospital and is being performed on melanoma and breast cancer patients.¹¹⁹ However, due to cost restraints the recommended serial-sectioning of SNs (see section 1.3) cannot be performed at Groote Schuur Hospital. Therefore, the histopathological evaluation of any SN tissues at this institute is likely to result in a significant underestimation of the presence of micro-metastases.

Molecular staging of the SN has the potential to facilitate and improve nodal tissue evaluation and the detection of metastases that would otherwise be missed by histopathology. Unfortunately, molecular evaluation has been viewed with some scepticism because the technique has been reported to be too non-specific.¹¹² In this study much emphasis was therefore placed on addressing this criticism. The overall aim

of this thesis was to develop a reproducible multi-marker RT-PCR assay with emphasis on achieving high specificity for the accurate detection of melanoma metastases in nodal tissue. Results from this study will lay the groundwork for long-term assessment of the clinical significance of the molecular detection of SN micro-metastases, as compared to IHC evaluation.

1.7 GENERAL AND SPECIFIC AIMS OF THE RESEARCH PROJECT

- ❖ **To develop an efficient, practical, and cost-effective protocol for the handling and homogenisation of lymph nodes, and the extraction of RNA from these tissues**

- ❖ **To develop a reverse transcription (RT) and single-stage, multi-marker polymerase chain reaction (PCR) assay for the specific and sensitive detection of melanoma cells in nodal tissue. In particular, the following aspects were addressed in order to achieve this aim:**
 - **Selection of molecular markers:** A multi-marker approach was pursued in order to improve specificity and detection rate.
 - **Selection of primers for single-stage PCR for each marker:** A single-stage PCR assay was developed for each marker in order to reduce the risk of amplicon contamination often associated with 2-stage PCR reactions (e.g. nested PCR).
 - **Control for RNA integrity:** A sensitive internal control marker was evaluated in order to minimise false-negatives caused by RNA degradation.
 - **cDNA-specificity assessment:** The specificity of the chosen primer pairs for cDNA was evaluated in order to exclude the amplification of contaminating DNA in the RNA sample.
 - **Multiplex PCR:** To develop multiplex PCR reactions (i.e. the co-amplification of markers) in order to reduce assay cost
 - **Optimisation of PCR cycle number for each marker:** In order to exclude the detection of unwanted transcripts in nodal tissue, yet still maintain the highest possible marker sensitivity, the PCR cycle number for each marker was carefully optimised.

- ❖ **To differentiate between melanoma cells and melanocytic nevi within nodal tissue in order to reduce the risk of false-positives.**
- ❖ **To assess the detection rate of each marker for the presence of melanoma cells in nodal tissue.**
- ❖ **To assess the sensitivity of each marker for the presence of melanoma cells in nodal tissue.**
- ❖ **To determine the reproducibility of the entire protocol.**

Chapter 2

MATERIALS AND METHODS

2.1 Sources and uses of human tissues

Cell lines: Eight melanoma cell lines were used (UCT-Mel-1 to -8), these having been established in the Department of Clinical Science and Immunology (University of Cape Town) from patient tumours as described before.²¹⁷ UCT-Mel-1 and -2 have been classified as pigmented, and UCT-Mel-3, -4, -5, -6, -7 and -8 as non-pigmented adherent cell lines according to the visual presence of pigment. The UCT-Mel-1 cell line was used to optimise the RNA extraction and RT-PCR assay, as well as for the spiking experiments to assess the sensitivities of each marker. All eight melanoma cell lines were used to assess the *in vitro* detection rate of each marker.

The Jurkat T (JT) human leukaemic cell line, obtained from the American Type Culture Collection, was used to provide background RNA into which UCT-Mel-1 RNA was mixed. This was then used to optimise various parameters in the RT-PCR protocol.

Lymph nodes: Two different types of lymph nodes were obtained:

(i) Melanoma-involved, lymph node sections (from enlarged/palpable nodes) from melanoma patients. These nodes were confirmed (by standard pathology) to contain malignant melanoma cells. The node sections were used as positive controls to assess the *in vivo* marker detection rate of each marker. Four node sections from four patients were obtained, each weighing more than 1.5 g. Informed and written consent was obtained from all the patients (see A.21 for sample of consent form).

(ii) Normal lymph nodes (visceral) from organ donors were used as negative control tissues. Twenty mesenteric nodes from two donors (10 nodes from each) were obtained, each node weighing less than 0.2 g. Informed and written consent was obtained from the next of kin.

Skin: One biopsy specimen from the forearm of a normal control subject was obtained from the Dermatology Department (Groote Schuur Hospital) and was used as another form of control tissue, where the presence of melanocytes would assess the ability of the multi-marker assay to differentiate between nodal neval cells and malignant melanoma

cells. This was necessary because the presence of nodal nevi/melanocyte clusters may lead to some false-positive RT-PCR results. Informed and written consent was obtained.

2.2 Cell culture growth conditions and preparation of cells for RNA extraction

The cells were grown in 100 mm Petri-dishes (Corning, Acton, USA) and maintained in RPMI 1640 medium (Sigma, St. Louis, USA) supplemented with 5% fetal calf serum (Highveld Biological, Johannesburg, SA), 50 IU/ml penicillin, and 20 µg/ml streptomycin at 37°C under 5% CO₂/95% air and 90% humidity. The complete growth medium is abbreviated as RP5. The medium was changed twice weekly (see A.11-A.14).

For RNA extraction, 2-3 sub-confluent melanoma cell culture dishes (approximately 2.5 x 10⁶ cells/dish) were washed once with phosphate-buffered saline (PBS), followed by versene-buffer for 10 mins at 37°C. The cells were then dislodged in versene-buffer with repetitive pipetting (large bore tip), and transferred into separate 10 ml sterile, polystyrene centrifuge tubes. The cells were centrifuged at 700 g for 5 mins at room temperature. The cell pellets were combined and resuspended in 5 ml RP5 medium. The cell number was determined using a haemocytometer. The cell suspension was centrifuged (700 g, 5 mins), and the supernatant aspirated to leave the cell pellet ready for RNA extraction. Approximately 2.5 x 10⁵ cells were replated per 100 mm tissue culture dish for routine sub-culturing (see A.15 and A.16).

JT suspension cell cultures were grown as for the melanoma cell cultures (see above). For RNA extraction, the contents of 2-3 sub-confluent JT culture dishes were pooled into a 50 ml sterile tube and the cells were pelleted by spinning at 700 g for 5 mins at room temperature. The medium was removed and the cell pellet was resuspended and washed in PBS. The cells were repelleted, and the supernatant aspirated to leave the cell pellet ready for RNA extraction.

2.3 The handling, homogenisation, and RNA extraction of lymph node tissue

Each lymph node (or part thereof) was placed in a sterile container and kept on ice immediately after surgical removal. If the node weighed less than 0.15 g it was placed directly into a labelled, sterile 2 ml polypropylene cryovial (Greiner Labortechnik, Frickenhausen, Germany). Each specimen weighing more than 0.15 g was rapidly

dissected on a sterile Petri-dish placed on a very cold surface (4°C). This was done since the recommendation for the RNA isolation reagent used i.e. TriPure (Boehringer Mannheim, BM, Mannheim, Germany) is not more than 0.1 g solid tissue per 1 ml TriPure, with the cryovial's maximum volume being approximately 1.5 ml (with the ball and tissue). A sterile blade and forceps (see A.2) were used for each lymph node. Large amounts of extra-nodal fat were first dissected off. The remaining tissue was dissected into pieces weighing approximately 0.15 g, and each then placed in a labelled sterile 2 ml polypropylene cryovial. The vials with tissue were snap-frozen in liquid nitrogen and stored at -75°C . The maximum time to elapse between surgical removal of the node and the freezing of the tissue was 2 hours. The tissue dissection was performed in a room separate from that used for gel electrophoresis.

On resuming the nodal processing, a cryovial containing a nodal piece was removed from the freezer and a sterile 8 mm stainless-steel ball (Bearing Man, Maitland, SA), as well as 1.5 ml cold TriPure was immediately added. The vial was then closed and attached to a Tekniva homogeniser (Tekniva, Cape Town, South Africa). The homogeniser breaks down tissue by providing high-speed (50 Hz), single plain harmonic motion to the metal ball located in the tube (International patent pending). The diameter of the ball was carefully selected to give maximum disruption of tissue between the ball and sidewalls of the vial. The homogeniser was switched 'on' for 5 seconds, followed by 10 seconds 'off', to prevent overheating of the sample. Effective tissue homogenisation usually required repeating the 'on-off' cycle 5-8 times.

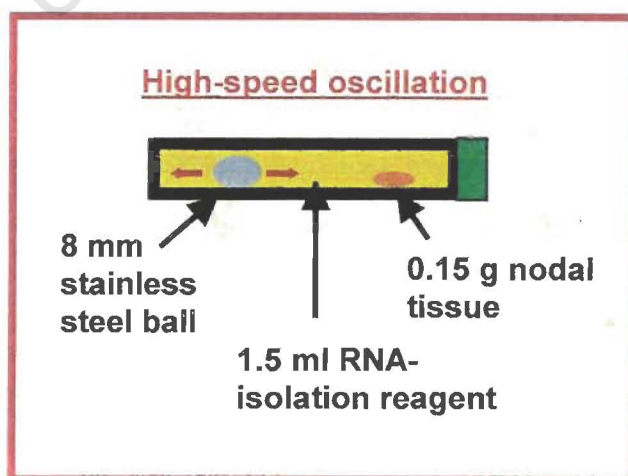


Figure 2.1 Schematic representation of the process of homogenising the nodal tissue The following oscillation parameters are adjustable: frequency, amplitude, duty cycle (on/off cycle time ratio) and total time on.

Half (750 μ l) of the homogenate was then transferred to each of two, 2 ml microfuge tubes. The cryovial tube, still containing the stainless-steel ball, was closed and disposed of appropriately for each lymph node. The RNA extraction was as described for cell cultures (see A.5). The two resultant RNA pellets were finally resuspended and pooled in a total of 100 μ l DEPC-treated ddH₂O, aliquoted and stored at -75°C . The RNA concentration was determined by spectrophotometry (see Appendix A.5). RNA gels were not performed since RT-PCR for the internal control marker was used to confirm the presence of intact RNA.

Skin: The entire biopsy specimen was stored at -75°C in a 1.8 ml cryovial. RNA was extracted from the skin biopsy as described above for nodal tissue.

2.4 Reverse Transcription (RT)

The reverse transcriptase enzymes sold by Applied Biosystem's were chosen because their buffers were identical to that of the AmpliTaq Gold™ polymerase (Applied Biosystems, AB, New Jersey, USA), which was the enzyme of choice for the PCR. (see section 2.5 below) The final reaction volume was kept at the lower range, to reduce reagent costs but yet allow for the possibility of the need to repeat a gel run.

Two specific aspects of the RT reaction were initially addressed. Firstly, the effect of variations in RNA template concentrations (4 μ g, 2 μ g, 1 μ g, 0.5 μ g and 0.1 μ g) on the RT-PCR efficiency was assessed. Secondly, the RT reaction was optimised with respect to cDNA extension time (60, 45, 30 and 15 mins). Both of these parameters were addressed by making use of UCT-Mel-1 RNA. The PCR parameters used were those as described below in section 2.5, with tyrosinase primers and 30 PCR cycles.

Each RT reaction (20 μ l total volume) was performed as follows (see A.8): The 10 μ l RT-mix consisted of 1x PCR-buffer (10 mM Tris-HCl, pH 8.3; 50mM KCl) (AB), 5 mM MgCl₂ (AB), 1 mM of each deoxyribonucleotide triphosphate (dNTP)(GibcoBRL-Life Technologies, Rockville, USA), 50 pmoles of Oligo(dT)₂₀ poly(A⁺) primer (University of Cape Town Biochemistry Dept., Cape Town, SA), 20 IU of MuLV Reverse Transcriptase (AB) and 10 IU of RNase Inhibitor (AB) per reaction. Then 0.2 μ l of DEPC-treated ddH₂O was added per reaction to allow for pipetting losses. The RT-'pre-mix' (excluding the enzymes) for each set of reactions was always prepared before thawing the RNA

sample. This 'pre-mix' was vortexed and pipetted repeatedly to ensure thorough mixing of the constituents, and centrifuged momentarily. This was kept at room temperature until the RNA was prepared. The RNA samples were thawed at room temperature. RNA samples were thawed once only and any residual RNA was discarded. A dilution of this RNA was made with DEPC-treated ddH₂O to a concentration of 0.1 µg/µl. This dilution was then heated at 90°C for 5 mins on the denaturing block of the robocycler (Stratagene, La Jolla, USA) to denature the secondary structure of the RNA strands. Then the samples were placed on ice for 5-10 mins.

The MuLV Reverse Transcriptase and the RNase Inhibitor were added to the RT-mix. Then 10 µl of the denatured RNA dilution was added to 10 µl of RT-mix. This was gently vortexed or pipetted to mix the enzymes, and aliquoted into the RNA samples. Each sample was overlaid with two drops of mineral oil using a 1 ml pipette tip. Complementary DNA was synthesised at 42°C on the annealing block of the robocycler for 30 mins, followed by 5 mins at 95°C on the denaturation block. The samples were then placed on ice for at least 5 mins. If the samples were to be used for Polymerase Chain Reaction at a later stage, they were stored at -20°C.

2.5 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (25 µl total volume) was performed as follows (see A.9): The 20 µl PCR-mix consisted of 1x PCR-buffer (10 mM Tris-HCl, pH 8.3; 50mM KCl) (AB), 1.5 mM MgCl₂ (AB) and 25 pmoles of each forward and reverse primer (University of Cape Town Biochemistry Dept., Cape Town, SA). DEPC-treated ddH₂O was added to make this volume up to 19.2 µl/reaction (incl. an extra 0.2 µl/reaction for pipetting losses). This PCR 'pre-mix' was vortexed and/or pipetted well and briefly centrifuged. Then 2 IU of AmpliTaq Gold™ (AB) was added and the pre-mix was vortexed briefly and/or pipetted again.

If the cDNA samples had been stored prior to use, they were allowed to thaw at room temperature. The cDNA was pipetted repeatedly beneath the mineral oil prior to aliquoting it into the PCR-mix. Then 5 µl of cDNA (equivalent to 0.25 µg RNA) was transferred to the 20 µl PCR-mix, which was then pipetted well. Of note is that the PCR-mix did not include any dNTP's since the required amount was transferred from the RT-mix, providing a final dNTP concentration of 200 µM per PCR reaction. A higher

concentration of dNTP's (400 μ M) consistently proved inhibitory to the PCR reaction. This effect was minimal after 30 PCR cycles, but significant after 60 cycles.

Each 25 μ l cDNA/ PCR-mix sample was overlaid with two drops (approximately 50 μ l) of mineral oil (using a 1 ml Gilson tip), and then cycled on the robocycler as follows: The first PCR cycle involved an initial denaturation at 96°C for 10 mins to activate the Taq Polymerase, annealing at 65°C for 2.5 mins, and extension at 72°C for 2 mins. This was followed directly by 29-59 PCR cycles (depending on the molecular marker and the experimental purpose) at 96°C for 30 seconds, 65°C for 2.5 mins, and 72°C for 2 mins. A final extension was done at 72°C for 10 mins to allow full extension of PCR products. The reaction was stopped on the cold block (6°C) for 5 mins and then stored at -75°C until analysed on a 2% agarose mini-gel (containing ethidium bromide) (see A.18).

The node preparation/dissection, RNA extraction, and RT and PCR reactions were all performed in a separate room from where gel electrophoresis was performed. Tubes containing PCR products were opened and gel electrophoresis was always performed under negative atmospheric pressure to minimize subsequent amplicon contamination of nodal/RNA samples or RT-PCR reagents.

2.6 Messenger RNA (mRNA) versus total RNA (τ -RNA)

Two micrograms, 5 ng and 50 pg of τ -RNA from the UCT-Mel-1 cell line was mixed with 40 μ g of JT τ -RNA to give the equivalent of 4×10^5 , 10^3 and 10^1 melanoma cells in a background of 8×10^6 JT cells respectively. From each of these samples, 2 μ g τ -RNA was removed and used for RT-PCR. Messenger RNA was extracted from the remaining τ -RNA (approximately 38 μ g) of each of these 3 samples using a biotinylated oligo-dT₂₀ primer (BM) and streptavidin-coated paramagnetic particles (BM), as described by the manufacturer (see A.6). The PCR parameters were as described above, and tyrosinase primers were used with 60 PCR cycles. The amount and specificity of the PCR products generated off the τ -RNA template versus the mRNA template were compared using agarose gel electrophoresis.

2.7 Selection of molecular markers

Using the United States National Library of Medicine PubMed search engine, a thorough computerised literature search, using the English language, was conducted on all

molecular markers previously used for the detection of melanoma metastases in the blood and lymph nodes. Marker detection rate and specificity were the main criteria used for marker selection.

2.8 Selection of primers for each marker

Pittman et al. (1996)⁷⁶ previously reported a single-stage RT-PCR assay for tyrosinase which was as sensitive as the previously described nested (2-stage) RT-PCR method⁵⁹ and which had the advantage of reducing the amplicon contamination risk. This approach was adopted for each marker in this study. The tyrosinase primers used were as published by Pittman et al. (1996).⁷⁶ (see Table 2.1)

TABLE 2.1 Selected tissue-specific markers

Primer	Primer sequence from 5' to 3' end	Reference
<HTyr 2	AGG CAT TGT GCA TGC TGC TT	59
>HTyr 3	GTC TTT ATG CAA TGG AAC GC	59
>Pmel-17/a	GCT TGG TGT CTC AAG GCA ACT	88
<Pmel-17/a	CTC CAG GTA AGT ATG AGT GAC	88
>Pmel-17/b	TGC TGG AGA GGT GGT CAA GTG	#
<Pmel-17/b	GGA GGG CAA AGG TCA GAG GCT	#
>MART-1/a	ATG CCA AGA GAA GAT GCT CAC	78
<MART-1/a	AGC ATG TCT CAG GTG TCT CG	78
>MART-1/b	CAC TCT TAC ACC ACG GCT GA	78
<MART-1/b	AGG TGA ATA AGG TGG TGG TGA	78
>MAGE-3/a	GAA GCC GGC CCA GGC TCG	74
<MAGE-3/a	GGA GTC CTC ATA GGA TTG GCT CC	74
>MAGE-3/b	TGA GGA GGC AAG GTT CTG AGG	#
<MAGE-3/b	GGT AGT GGG GAG GCT GGA GGC T	#

#Unpublished primers for Pmel-17 and MAGE-3, designed by Hanekom and co-workers using *DNAMAN 5.1.0.0* (Lynnon Biosoft).

PMel-17, MART-1 and MAGE-3 were the other markers selected on the basis of a thorough literature search (see *Results and discussion*, section 3.5.1). Nested PCR primers previously used for these markers (see Table 2.1) were tested in different

combinations to select the best set of primers for single-stage PCR. For example, >Pmel-17/a was tested in combination with <Pmel-17/a and <PMel-17/b, and so also >PMel-17/b was tested in combination with <Pmel-17/a and <PMel-17/b. The best primer combination for each marker was chosen on the basis of optimum product yield and specificity. RNA from the tyrosinase-expressing UCT-Mel-1 cell line⁶³ was used as the RT-PCR template for the purpose of selecting the PMel-17, MART-1 and MAGE-3 primers, as it was found to express all four markers. The PCR parameters used for all four markers were as described above (with 35 PCR cycles), with the exception of the annealing temperature, which was 55°C.

2.9 Internal control of RNA integrity and RT efficiency

The Porphobilinogen deaminase (PBGD) housekeeping gene, reported to be expressed at low levels per cell,⁷⁸ and the commonly used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene were compared to confirm that the former would provide a more sensitive internal control for RNA integrity. The primers used for PBGD and GAPDH were as described in Table 2.2. The expected band sizes were 339 bp and 600 bp for PBGD and GAPDH respectively.

TABLE 2.2 Internal control markers compared

Primer	Primer sequence from 5' to 3' end	Reference
>PBGD	CTG GTA ACG GCA ATG CGG CT	78
<PBGD	GCA GAT GGC TCC GAT GGT GA	78
>GAPDH	ACG GAT TTG GTC GTA TTG GG	63
<GAPDH	AGG GAT GAT GTT CTG GAG AG	63

The markers were compared by titrating the amount of RNA used (1, 0.5 and 0.1 µg) in the RT reaction. RNA from the UCT-Mel-1 cell line was used for this purpose. The parameters were as described above (with 35 PCR cycles), with the exception of the annealing temperature which was 60°C. All the primers were synthesised by the Department of Biochemistry (University of Cape Town Biochemistry Dept., Cape Town, SA) using an automated process.

2.10 Optimisation of annealing temperature

After selecting the best primer sets, an annealing temperature gradient (55°C-65°C) was performed for each marker (including the PBGD primers). This was done in order to determine the annealing temperature for each marker that would produce the highest specificity and product yield. RNA from the UCT-Mel-1 cell line was used for this purpose.

2.11 Positive and negative controls

An H₂O-negative control was performed concurrent with each RT-PCR run to exclude false-positives that may result from contamination of RT-PCR reagents with cDNA or amplicons. This was done by replacing the RNA-template of the RT reaction with DEPC-treated ddH₂O. Positive controls using UCT-Mel-1 RNA as RT-PCR template were performed with each assay on a nodal sample. This served to confirm a flawless RT-PCR reaction, in that none of the reagents were accidentally omitted, and that the reaction parameters were not altered.

2.12 cDNA-specificity assessment

In order to ensure that amplification of residual genomic DNA in the RNA sample will not occur during RT-PCR, it is essential that the PCR primers are assessed for cDNA specificity. The chosen set of forward and reverse primer for each marker was assessed for cDNA specificity, firstly, by examining their positions in the respective mRNA sequence, as obtained from the GenBank database (<http://www.ncbi.nlm.nih.gov>). Specifically, the presence of one or more large intronic sequences between each forward and reverse primer, and the positioning of any of the primers across an intron/exon boundary, were looked for. Secondly, cDNA-negative controls were performed to exclude genomic DNA amplification as a cause of false-positives. This was done by omitting the reverse transcriptase from the RT-step, followed by PCR amplification for each marker. RNA from the UCT-Mel-1 cell line was used for this purpose. The PCR parameters were as described above, with 40 PCR cycles.

2.13 Optimisation of PCR cycle number to obtain maximum specificity for each marker

This was determined as the maximum PCR cycle number (for maximum sensitivity) at which 100% specificity for the presence of melanoma cells in normal nodal tissue could be achieved for each marker. Each marker was initially tested on the RNA derived from 10 normal lymph nodes at 50, 40 and 30 PCR cycles. The PCR cycle number at which no false-positives were generated was chosen for each marker. If false-positives were detected at 40 PCR cycles, the nodes were re-evaluated at 35 cycles (as opposed to 30 cycles) so as not to compromise sensitivity too much. RNA from a further 10 normal lymph nodes was then evaluated to confirm the specificity of each marker at the chosen PCR cycle number.

2.14 Assessment of the sensitivity of each marker

This sensitivity assessment was based on the expression levels of the four markers in the UCT-Mel-1 cell line. A sub-confluent culture of this cell line was removed from the Petri-dish and counted using a haemocytometer. Ten times serial dilutions of the cells were prepared in RP5 medium, starting at 5×10^4 cells/ml down to 5×10^1 cells/ml. Then 200 μ l of each dilution was placed in a micro-titre well to provide 10^4 , 10^3 , 10^2 and 10^1 cells per well. The lowest dilution (10^1 cells/well) was plated in quadruplet to allow selection of a well containing approximately 10 cells, as verified by microscopy (40x magnification). The cells were allowed to settle for 30 mins at 37°C in the CO₂ incubator. Concurrently, normal lymph node tissue (equivalent to 0.4 g in total) was homogenised according to the previously described protocol (see section 2.3). The homogenates were pooled and four 1 ml aliquots were placed in 1.5 ml microfuge tubes. The contents of each micro-titre well (containing the serial dilutions of the UCT-Mel-1 cells in 200 μ l RP5 medium) was then transferred to 1 ml of nodal homogenate. Each well was thoroughly rinsed out with 200 μ l of the respective nodal TriPure homogenate, and then again with 200 μ l of fresh TriPure, this being transferred back to the respective microfuge tubes. The RNA extractions were completed, as described in A.5. This resulted in RNA from a known number of melanoma cells being present in background RNA derived from 0.1 g of normal lymph node tissue.

2.15 Co-amplification of markers (multiplex PCR)

In order to minimise the cost of the assay, it was decided to investigate whether markers with the same optimal PCR cycle number i.e. MART-1 plus MAGE-3 at 35 cycles, and Pmel-17 plus PBGD at 30 cycles (see *Results and discussion*, section 3.5.4), could be amplified in the same reaction (i.e. multiplex PCR). This was possible since the relevant primer combinations had the same optimal annealing temperatures (i.e. 65°C) and their product sizes were different enough to allow easy distinction on agarose gel electrophoresis. It was important though, to first assess whether co-amplification of the markers would affect the sensitivity and specificity of each marker. This was achieved by comparing the multiplex PCR products with the products obtained when each marker was amplified individually (by using the cDNA samples as in section 2.14). The amount of H₂O used per PCR reaction was reduced to maintain the total PCR reaction volume at 25 µl (see A.9). Tyrosinase was kept as a stand-alone marker at 40 cycles.

2.16 Assessment of the detection rate of each marker

The *in vitro* and *in vivo* detection rates for melanoma cells were assessed for each marker by evaluating 8 melanoma cell lines and 4 melanoma-involved lymph nodes respectively. This was done using multiplex PCR for MART-1 plus MAGE-3 at 35 cycles, Pmel-17 plus PBGD at 30 cycles, and tyrosinase amplified alone at 40 cycles.

2.17 Will this multi-marker assay allow differentiation between melanoma and neval cells within a node?

Nodal melanocytic cell clusters/nodal nevi are reported to be present in approximately 4% of melanoma-draining lymph nodes (see *Literature Review*, section 1.4.2.3). In order to determine whether any of the markers would differentiate between melanocytic nevi and melanoma cells, the expression of the markers in a melanoma-involved node and skin melanocytes (simulating nodal neval cells) was compared. Pigmented skin (containing melanocytes) was used as the control tissue since appropriate control nodes are practically unobtainable (see *Results and discussion*, section 3.6)

CHAPTER 3

RESULTS AND DISCUSSION

In view of the technical nature of this project, the results and discussion are presented together. Part 1 covers the basic parameters of the RT and PCR reactions that were optimised. Part 2 covers the optimisation of assay parameters that are critical for the development of a highly accurate molecular assay for the detection of melanoma nodal metastases.

PART 1

3.1 OPTIMISATION OF THE REVERSE TRANSCRIPTION (RT) REACTION

It has been shown that the RT-step is the main source of variability in RT-PCR efficiency.^{89, 184} Three RT parameters were examined in order to ensure that maximum cDNA extension might reproducibly be achieved for every patient sample: These were RNA concentration, RT extension time and the use of total RNA versus messenger RNA.

3.1.1 The effect of varying RNA concentrations on RT-PCR efficiency

Theoretically, it is possible that an increase in the specific mRNA template concentration would result in a greater degree of sensitivity of the RT-PCR assay. However, it has been reported that high RNA concentrations can inhibit the efficiency of the RT-PCR reaction,²¹⁸ although another study found no variation within the range of 1-6 µg total RNA as template.⁸⁹

It is difficult to accurately measure the RNA yield from every RNA extraction by spectrophotometry because the reading does not take DNA contamination into account.²¹⁹ Therefore, in order to establish whether any variations in RNA concentration would significantly alter the RT-PCR efficiency, and also to ensure that maximum sensitivity would be achieved, different quantities of RNA from a melanoma cell line (UCT-Mel-1) were subjected to RT-PCR using primers specific for tyrosinase. The RT-PCR products obtained from 4 µg, 2µg, 1 µg, 0.5 µg and 0.1 µg of this RNA (Figure 3.1, lanes 1-5) were found to be essentially identical. This indicates that variations of RNA concentration in this range did not affect the efficiency of RT-PCR amplification, and that

the use of 1 μ g RNA per RT reaction provided the same sensitivity as higher concentrations of RNA.



Figure 3.1. The effect of different RNA template concentrations on RT-PCR efficiency for tyrosinase amplification. Lane M, 100 bp molecular marker. Lanes 1-5, RNA concentrations of 4, 2, 1, 0.5, and 0.1 μ g. Lane 6, negative (H_2O) control.

3.1.2 Optimisation of the cDNA extension time

The effect of different cDNA extension times on RT-PCR yield was next investigated in order to establish conditions under which maximum product yield might be achieved. Most RT protocols suggest a cDNA extension time of 45–60 mins. The manufacturer of the RT-enzyme used (i.e. Applied Biosystems) recommends 15 mins. Therefore, cDNA extension times of 15, 30, 45 and 60 mins were compared by using UCT-Mel-1 RNA and PCR primers specific for tyrosinase.

No difference in RT-PCR product yield was detectable for cDNA extension times of 30, 45 and 60 mins (Figure 3.2, lanes 2-4). Slightly less PCR product was obtained after a 15 min cDNA extension time (lane 1), although this is not clear on the print of the gel. Therefore a 30 min cDNA extension was selected as the standard.

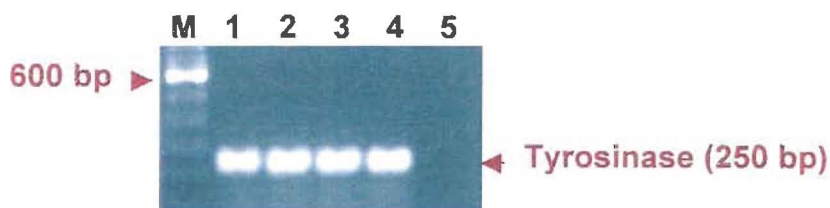


Figure 3.2. Optimisation of cDNA extension time using tyrosinase primers. Lane M, 100 bp molecular marker. Lanes 1-4, cDNA extension times of 15, 30, 45 and 60 mins. Lane 5, negative (H_2O) control.

3.1.3 Re-assessment of cDNA extension time

It was shown above that a minimum of 30 mins was necessary for maximum RT-PCR yield. However, this assessment was performed on RNA samples from melanoma cells

that contain abundant tyrosinase mRNA template. The limitation of this experimental design is that any differences in yield between samples undergoing increasing extension times might be masked by the abundance of PCR products generated in each sample (see Figure 3.2 above). Thus, in order to more accurately determine the effect of different cDNA extension times on RT-PCR yield, the following experiment was carried out: Five identical samples of RNA from a normal node homogenate spiked with 10 UCT-Mel-1 cells (see *Materials and Methods*, section 2.14) were each subjected to RT with different extension times (i.e. 5, 10, 15, 30 and 60 mins). This was followed by 40 PCR cycles for tyrosinase and 30 cycles for PBGD.

The results showed that a 15 min cDNA extension time was sufficient for maximum tyrosinase yield (Figure 3.3, lane 3). However, for the housekeeping gene Porphobilinogen deaminase (PBGD) (see section 3.4), it would seem that longer extension times (30-60 mins, lanes 9 and 10) are needed for maximum product yield.

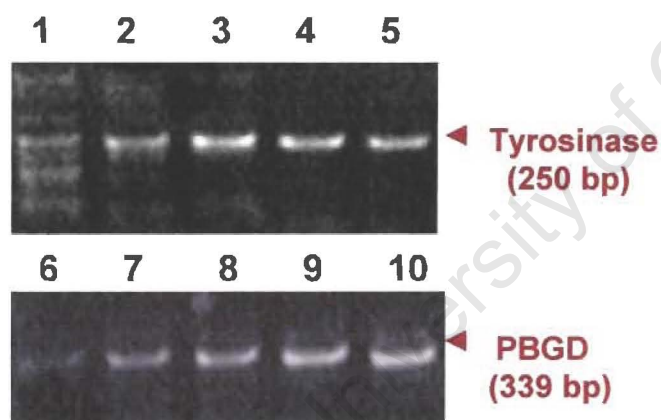


Figure 3.3 Re-assessment of optimal cDNA extension time. Lanes 1-5, cDNA extension times of 5, 10, 15, 30, 60 mins for tyrosinase. Lanes 6-10, cDNA extension times of 5, 10, 15, 30, 60 mins for PBGD. The results are representative of two independent experiments.

3.1.4 Messenger RNA (mRNA) versus Total RNA (τ RNA)

It has been reported that performing RT-PCR on an mRNA fraction instead of τ RNA results in an increase in assay sensitivity as well as specificity.²²⁰ The next step was therefore to determine whether this would indeed be the case for the tyrosinase RT-PCR assay. In order to accurately do this, varying amounts of melanoma τ RNA were spiked into constant amounts of τ RNA from a Jurkat-T (JT) leukemic cell line (a non-melanoma cell line) (see *Materials and Methods*, section 2.6).

The results showed that the use of the mRNA extracted from the equivalent of 10^1 or more UCT-Mel-1 cells (Figure 3.4, lanes 6, 4 and 2) did not prove to be more sensitive or specific than performing RT-PCR on 1 μg of τRNA of the same samples (Figure 3.4, lanes 5, 3 and 1). This result is difficult to explain since 20x more mRNA template should have been present in each of the final mRNA extracts compared to the samples with τRNA . Moreover, the removal of the bulk of non-target RNA (i.e. transfer and ribosomal RNA) during mRNA extraction should theoretically have contributed to a more sensitive assay when using mRNA. However, since the comparative analysis in this study did not (repeatedly) show an increase in RT-PCR sensitivity or specificity when using mRNA as template, this extra labour-intensive step, which also introduced extra costs and was prone to the introduction of technical inconsistencies, was abandoned.



Figure 3.4. The use of mRNA versus τRNA . Lanes 1, 3, 5 and 7: PCR products using 2 μg τRNA as template. Lanes 2, 4, 6 and 8: PCR products using the mRNA fraction, extracted from approximately 38 μg τRNA as template. Lanes 1 and 2: 2 μg UCT-Mel-1 τRNA in 40 μg JT τRNA . Lanes 3 and 4: 5 ng UCT-Mel-1 τRNA in 40 μg JT τRNA . Lanes 5 and 6: 50 pg UCT-Mel-1 τRNA in 40 μg JT τRNA . Lanes 7 and 8: JT τRNA only. The results are representative of at least two independent experiments.

3.2 OPTIMISATION OF THE POLYMERASE CHAIN REACTION (PCR) PARAMETERS

3.2.1 Optimisation of annealing temperature for each marker

In order to ensure the highest PCR product specificity and maximum product yield, an annealing temperature gradient was performed for each of the chosen primer sets, as listed in Table 2.1. For all the primer sets, the amplification products were essentially identical over the range of annealing temperatures tested (55°C to 65°C) for each marker. Figure 3.5 shows the results from the RT-PCR reactions using the primers for MAGE-3. The results for the other markers are not shown. It was therefore decided to use 65°C as the annealing temperature for all the markers since an improved specificity is generally obtainable at a higher annealing temperature. Furthermore, the use of

identical annealing temperatures was advantageous since it was envisioned that, in the future, combined (multiplex) PCR might be carried out.

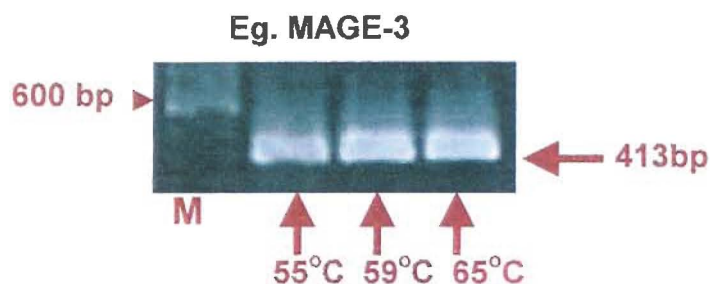


Figure 3.5. Optimisation of the annealing temperature for MAGE-3. M, 100 bp molecular marker.

3.2.2 Co-amplification of markers (multiplex PCR)

With multiplex PCR, two or more target cDNA sequences are co-amplified by including more than one primer set in the same reaction tube. Multiplex PCR has the potential to produce considerable time, effort and cost savings, without compromising test utility.²²¹ If the internal control marker is included in a multiplex PCR reaction this could, in addition, facilitate future quantitative RT-PCR.²²² However, special attention must be given to primer design to reduce the chances of non-specific interactions occurring, which could also reduce sensitivity and give rise to non-specific PCR products. There is no means of predicting whether such non-specific primer interactions will occur, and a trial-and-error approach is necessary. Moreover, it has been shown that if PCR products are not of similar sizes, they will not amplify consistently when used in a multiplex PCR reaction.¹⁸⁶ Lastly, it is reported that co-amplification of high abundance housekeeping gene transcripts with low abundance gene transcripts is impossible.²²²

In order to determine whether some of the PCR primer sets selected in this study (see section 3.5.2) could be co-amplified, the RNA from nodal tissue spiked with decreasing numbers of melanoma cells was used, and the following primer pairs tested together: The MAGE-3 and MART-1 primers were first co-amplified, since the optimal PCR cycle number (for maximum specificity) for both of these markers was found to be 35 cycles (see section 3.5.4). Thereafter, the Pmel-17 and PBGD primers were co-amplified, since 30 PCR cycles was found to be optimal for both these markers (see section 3.5.4). (Co-amplification of the primer sets was made possible by the fact that all the primer sets had been found to amplify optimally at 65°C.)

By comparing the sensitivities achieved with the above multiplex PCR reactions (Figure 3.6), to the sensitivities achieved when the reactions were carried out separately i.e. uniplex PCR (see Figure 3.18, section 3.8), it was evident that co-amplification of these markers did not reduce the sensitivity or specificity for any marker. For example, MAGE-3 was detected in samples containing a minimum of 10^2 cells in a background of 0.1 g of nodal tissue, whether amplified in a multiplex reaction with MART-1 (Figure 3.6), or as a uniplex reaction (Figure 3.18).

Thus, three instead of five PCR reactions could be performed per assay using these five markers, this reducing the cost of reagents and disposables. Although further cost saving could be achieved by co-amplification of all 4 markers plus the internal control (results not shown), the different optimal PCR cycle numbers made this inappropriate, unless real-time PCR had been used. Also, two non-specific bands having similar sizes as the MAGE-3 and MART-1 products were intermittently visible with single-marker PBGD amplification (results not shown), and this could have potentially confused the interpretation of the results.

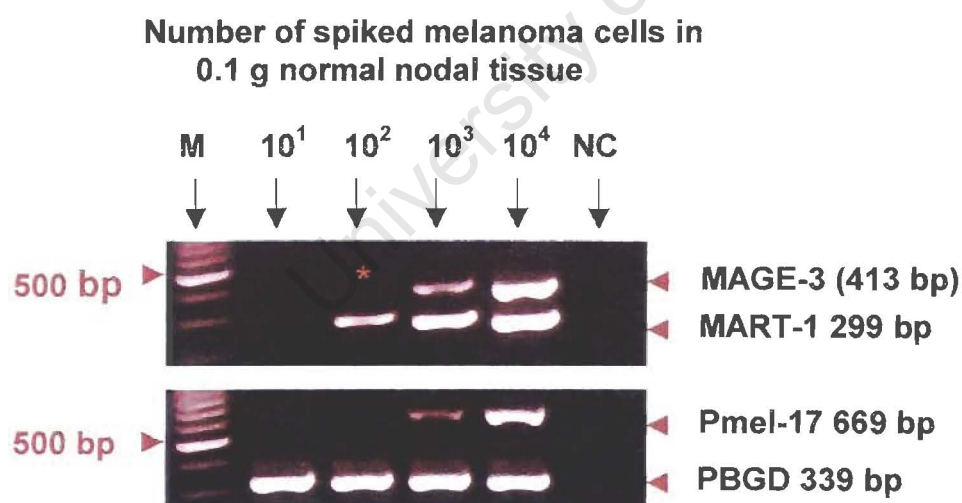


Figure 3.6. Assessing the sensitivity and specificity of the (MAGE-3 + MART-1) and (Pmel-17 + PBGD) multiplex PCRs. Lanes M, 100 bp molecular marker. Lanes NC, negative (H_2O) controls. The results are representative of two independent experiments.

* Note, MAGE-3 band for 10^2 melanoma cells not visible on print, but visible on original.

PART 2**3.3 THE HOMOGENISATION OF, AND RNA EXTRACTION FROM NODAL TISSUE**

Since the molecular analysis of tissues relies on the integrity of the RNA in the sample, it is vital that RNA degradation be limited in order to prevent false-negatives. In most studies the nodal tissue section(s) to be used for RT-PCR analysis is/are snap-frozen in liquid nitrogen and then disrupted into single cells by gentle scraping or mincing with a sterile scalpel, followed by homogenisation in a lysis buffer.^{131, 148} These homogenisation techniques tend to be impractical and are likely to cause significant RNA degradation. Other investigators make use of rotor-stator homogenisers.^{187, 188} These homogenisers (e.g. Ultra-Turrax, VWR Scientific Products, New Jersey, USA) cut, rip and pulverize solid tissues by direct mechanical action of the stainless steel blades, which can be changed and cleaned. This does, however, involve lab ware that requires stringent washing and decontamination of RNases.

One of the important aims of this study was thus to develop an efficient, cost-effective, practical method of nodal homogenisation that would overcome the above problems and would be easily standardised. The unique protocol, using high-speed oscillation of a metal ball that crushes the tissue, is detailed in *Materials and Methods*, section 2.3. This protocol has the following advantages over those methods used in previous studies. First, since the tissue is frozen only once and the cells thaw and lyse directly in the presence of guanidinium thiocyanate (in the TriPure), a potent RNase inhibitor, the risk of RNA degradation during the handling and homogenisation steps should be minimal. Secondly, the method used in this study allows the homogenisation of nodal tissue using disposable lab ware only, thus eliminating the need to sterilise lab ware between samples. The method therefore also reduces the risk of inter-sample contamination and is safe for the technician. Very importantly, the protocol is rapid and cost-effective, which further facilitates implementation of this protocol for routine clinical purposes. Finally, since a normal node weighs anything between 0.15-0.6 g,¹⁹⁴ the RNA from at least a quarter of a normal-sized node can be extracted in one homogenisation step. A new tube cradle is currently being designed that will be adaptable for cryovials of larger volume. Thus larger node sections, and even entire SNs submitted, could be processed in this way.

In this study, the relative consistency of spectrophotometric readings of the RNA extract (OD_{260}/OD_{280} ratios of approximately 1.7 for more than 50 different extractions) was reassuring of the efficiency and reproducibility of the RNA extraction step. The RNA yield per gram nodal tissue was relatively consistent: approximately 200 μg τRNA from 0.15 g of tissue. Melanoma cell culture RNA extractions yielded on average 15 μg of τRNA per 10^6 cells, with similar OD_{260}/OD_{280} ratios as the nodal extractions. The consistency of the OD ratio is important as the RT enzyme may be inhibited by residual salts, alcohol, phenol or protein contaminants. In order to critically evaluate the efficiency and reproducibility of the homogenisation and RNA extraction steps, a sensitive assessment of the reproducibility of the entire protocol was assessed later in this study, once all the RT-PCR parameters were optimised (see section 3.9).

3.4. INTERNAL CONTROL OF RNA INTEGRITY AND RT EFFICIENCY

If one wishes to detect micro-metastatic tumour deposits in nodal tissue, one must be aware that a false-negative result may result from RNA degradation that had occurred prior to, or during the RNA extraction and/or RT step. This would go unnoticed unless one used an internal control marker that enabled one to monitor the quality of the RNA for every cDNA sample. In addition, such a sensitive internal control could also be reflective of RT efficiency.

Most studies performing RT-PCR make use of the ubiquitously expressed house-keeping genes (e.g. glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -actin and β_2 -microglobulin²²³) as internal controls to monitor RNA integrity and RT-efficiency. If a cDNA sample is negative for the housekeeping gene selected, or the amount of house-keeping gene PCR product is visually less, then the RNA sample would usually be discarded and the RT-PCR repeated on another RNA aliquot. However these housekeeping genes are so abundantly expressed that they can still usually be detected from cDNA that has been produced from RNA of rather poor quality, with the result that false-negatives may still be undetected.¹⁰⁴

Since the aim of this study was to develop an assay that can accurately detect very few (less than 100) melanoma cells in a large background of nodal tissue, it was of concern that if even a small percentage of the RNA were degraded, specific melanoma transcripts might become undetectable. In such a case, the GAPDH product would most

likely still be detectable, thereby producing a false-negative result for the marker. In order to solve this problem an alternative internal control marker was sought. Porphobilinogen deaminase (PBGD) is a house-keeping gene that encodes a protein involved in the haem-biosynthetic pathway. It is ubiquitously expressed, but at significantly lower levels than GAPDH and has thus been used as an alternative control gene in many published studies.^{78, 224, 225} The use of this marker, as an alternative to GAPDH for the control of RNA integrity (and RT efficiency), was therefore assessed by using decreasing quantities of UCT-Mel-1 RNA. Figure 3.7 confirms that there are fewer PBGD mRNA template molecules per μg of UCT-Mel-1 RNA when compared to GAPDH, this having been previously reported by de Vries et al. (1999).⁷⁸ This confirms that PBGD is a more sensitive internal control than GAPDH.

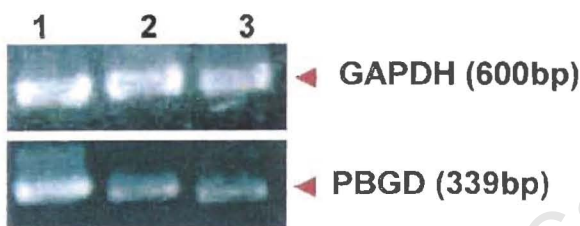


Figure 3.7. The comparison of GAPDH and PBGD expression in samples containing decreasing RNA concentrations. Lanes 1, 2 and 3, RT-PCR products with cDNA produced from 1, 0.5 and 0.1 μg of RNA. The results are representative of two independent experiments.

However, it remains to be established whether PBGD is sensitive enough to detect the minimum degree of RNA degradation needed to produce a false-negative result when micro-metastatic deposits are present in the SN. A possible way to address this issue would be to induce RNA degradation (in a controlled experimental manner) in normal nodal samples spiked with a few (e.g. 10-100) melanoma cells. If the tyrosinase signal disappeared with a limited amount of RNA degradation, yet the PBGD signal remained detectable, it would indicate that PBGD is inadequate to reveal limited RNA degradation. If this is the case, it might be worthwhile to search for an even more sensitive marker to assess nodal RNA integrity.

It has been suggested that a more sensitive evaluation of RNA degradation could be achieved by introducing a constant number (10^3) of Jurkat T (JT)-cells (these cells express the unique JT-cell receptor, i.e. TCRBV) into samples, at the RNA extraction step.²²³ RT-PCR with primers for TCRBV could then be used to monitor RNA degradation. Unfortunately, this is an extra labour-intensive step and moreover, it would

not reveal RNA degradation that may have occurred prior to RNA extraction. The ideal marker would be one that is expressed solely in a cell type consistently present in limited numbers in lymph nodes. One possibility is to use PMP22 (a glial cell-specific marker), which is expressed at high levels in Schwann cells.²²⁶ Since low numbers of Schwann cells are present in normal lymph nodes (see *Literature Review*, section 1.4.2.3), PMP22 mRNA could be a potential sensitive target to reveal limited RNA degradation.

It is important to note, however, that a positive result for the internal control marker does not ensure a flawless PCR reaction for the specific marker. Hence, in addition, a positive control (see *Materials and Methods*, section 2.11) is always necessary to confirm consistency in PCR cycling parameters and the functionality of the specific set of marker primers, as well as to detect errors in preparing the PCR reagent mix.

3.5 DEVELOPMENT OF A SINGLE-STAGE, MULTI-MARKER RT-PCR ASSAY

3.5.1 Selection of molecular markers

Heterogeneity of antigen expression is a feature of all malignant cells and becomes increasingly evident in advancing stages of tumour progression.⁸⁵ Therefore, although tyrosinase has been found to be expressed by most primary melanomas,^{87, 88, 95, 174} it is likely that tyrosinase expression will be absent in a proportion of metastases, including SN micro-metastases. This may account for some of the unpredicted nodal recurrences occurring in patients who were found to be SN-negative by single-marker tyrosinase RT-PCR.

Numerous studies making use of an RT-PCR assay for the detection of melanoma cells in the peripheral blood^{74, 87, 89, 92, 93} and in lymph node tissue^{87, 128, 152, 202} have shown that the detection rate of metastasising melanoma cells in these target tissues is significantly increased if one makes use of a multi-marker approach. Therefore, in order to develop an RT-PCR assay with an improved detection rate, and thus ultimately reduce the risk of false-negatives when evaluating the SN, a multi-marker approach was chosen. Based on a thorough review of the above studies, two other pigment cell-specific (PCS) markers (in addition to tyrosinase) were chosen, namely Pmel-17 and MART-1. MAGE-3, a cancer/testis antigen (CTA) was also included in the panel of markers.

Rationale for the selection of markers:

For the detection of circulating melanoma cells (CMCs), MART-1 and MAGE-3 in particular have been shown to improve the detection rate of tyrosinase RT-PCR in patients with advanced melanoma, and to be highly specific markers.^{73, 74, 78, 89-91, 93, 95} Although some investigators found Pmel-17/gp100 (a PCS marker) to be suitable,^{87, 95} others have reported it to be detectable in the blood of normal controls and non-melanoma cancer patients.^{73, 89}

The same molecular markers that have been assessed for the detection of CMCs have also been used for the evaluation of nodal tissue. In particular, MART-1,^{87, 128, 152, 202} Pmel-17^{87, 152, 187} and MAGE-3¹²⁸ have all been shown to improve the detection rate of single-marker tyrosinase RT-PCR for melanoma cells in nodal tissue. The detection rate of Pmel-17 is 79%⁸⁷ and of MART-1 between 86-100%,^{87, 128, 202} as evaluated on melanoma-involved nodes that were IHC-positive. Therefore, the use of such a combination of three PCS markers should significantly reduce the risk that metastatic melanoma deposits would be missed. In addition, the multi-marker approach is advantageous in that different patterns of marker expression might in the future provide valuable prognostic information.^{91, 92}

The decision to include MAGE-3 as a marker was not so much to improve detection rate, but rather to address the risk of false-positives due to the reported presence of nodal nevi in 8.5% of melanoma-draining SNs that are (histopathologically) negative for melanoma cells (see *Literature Review*, section 1.4.2.3). These neval cells have been shown to have a similar morphology and expression profile as that of normal skin melanocytes, and hence also melanomas, and are thus likely to express most, or even all of the PCS markers.^{61, 112, 128} Although the numbers of these neval cells within a lymph node are low, the extreme sensitivity of RT-PCR means that neval cell PCS transcripts (i.e. unwanted transcripts) could become detectable, especially with high PCR cycle numbers. Unfortunately, the origin of the PCS transcripts detected by RT-PCR cannot be confirmed to be melanoma cells, as is the case with histopathology (by using H&E re-examination). As a consequence, false-positives may occur when using RT-PCR for the PCS markers, and this risk has been raised as a strong argument against the use of RT-PCR for SN evaluation.

Therefore, with the aim being to allow differentiation between melanoma cells and the specific but unwanted PCS transcripts derived from SN neval cells, it was decided to search for, and then include, a marker that is reported as being expressed by melanoma cells and not by nodal neval cells. At the time of marker selection, the melanoma-associated antigen (MAGE) gene family, and in particular the MAGE-A genes, had been characterised and shown to be specific for malignant cells. These genes are now classified as cancer/testis antigens (CTA), since they are reportedly not expressed in any normal tissues other than testicular and placental cells.^{176, 178} MAGE-3 is a member of the MAGE-A gene family and has a reported detection rate of between 45-71% for nodal melanoma cells.^{128, 227} MAGE-3 was therefore selected for its potential to improve the assay's specificity for the detection of melanoma cells in nodal tissue.

3.5.2 Selection of primers for single-stage PCR for each marker

Most studies using RT-PCR for the detection of metastatic melanoma cells make use of a nested (2-stage) PCR approach. This typically involves an initial 30 PCR cycles using an 'external' set of primers (e.g. >Tyr1, <Tyr2), followed by another 30 PCR cycles (using an aliquot from the first reaction) using an 'internal' set of primers (e.g. >Tyr3, <Tyr4), as shown for tyrosinase in Figure 3.8.

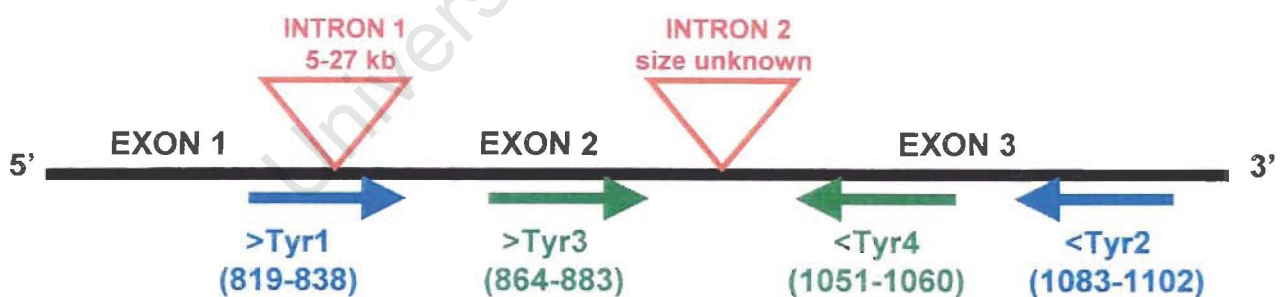


Figure 3.8. Schematic representation of the cDNA positions of the nested tyrosinase primers used by Smith et al. (genomic organisation and primer positions are according to the Genbank accession numbers AF237807 and Y00819)

The advantage of this 2-stage PCR is that a high degree of sensitivity can be achieved by increasing PCR product yield through the addition of fresh Taq polymerase after 30 cycles. However, because the PCR tube must be opened in order to add the fresh supply of Taq polymerase, there is an increased risk of releasing amplicons, which may then contaminate future reactions.

Therefore, in order to limit the risk of spurious false-positives due to amplicon contamination, in this study a single-stage PCR (i.e. one set of primers for each marker) was optimised for each marker. For tyrosinase, a previously reported single set of primers (see *Materials and Methods*, section 2.8) was tested for specificity (Figure 3.9). For MAGE-3, MART-1 and Pmel-17, various combinations of the available nested forward and reverse primers were tested as described in *Materials and Methods* (section 2.8) and the results shown in Figure 3.10.

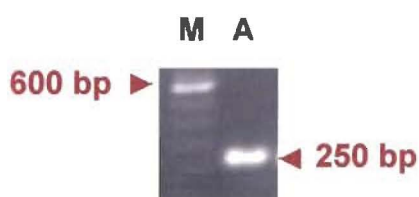


Figure 3.9. Tyrosinase single-stage PCR product using >Tyr3 and <Tyr2 primers. Lane M, 100bp molecular marker. Lane A, tyrosinase RT-PCR product

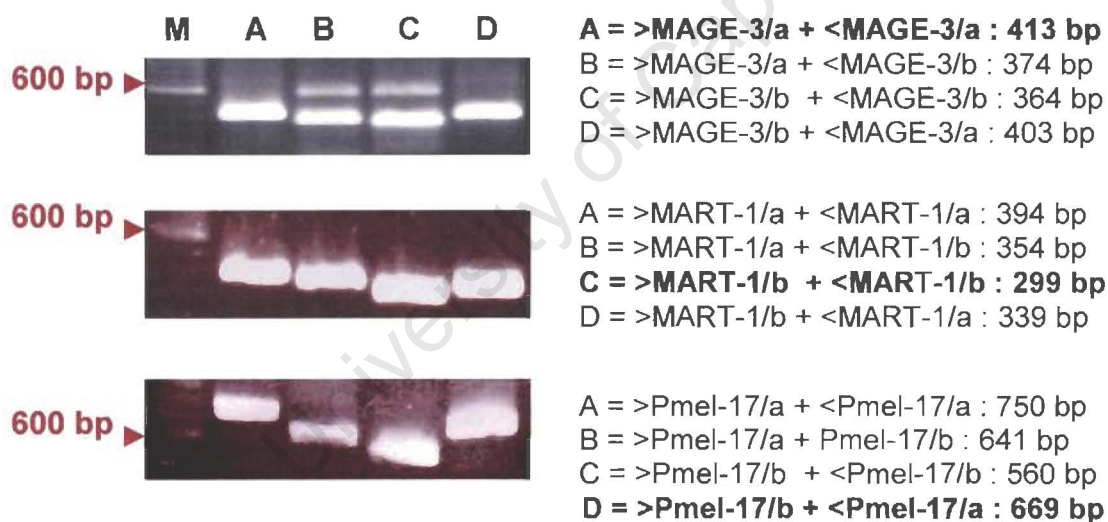


Figure 3.10. The different PCR products derived from the four different combinations of the available primers for MAGE-3, MART-1 and Pmel-17. Lane M, 100 bp molecular marker. The primer pair then selected for each marker has been bolded.

The tested tyrosinase primer set produced a very specific PCR product of 250 bp and was therefore used in the study for the detection of the presence tyrosinase mRNA. The most optimal single set of primers for MAGE-3, MART-1 and Pmel-17 was selected on the basis of specificity, comparative yield and size of the PCR product (each set being indicated in bold-type in Figure 3.10). The results for the different combinations of the MAGE-3 primers, in particular, clearly indicated that certain combinations result in non-

specific PCR products (i.e. Figure 3.10, lanes B and C for MAGE-3), and thus neither of these two primer sets would be suitable. PCR product size was also taken into consideration since a future objective was to develop multiplex PCR (i.e. amplification of more than one marker in the same PCR reaction tube) that would require sufficient product size differences (to allow the bands to be easily distinguishable on agarose gel).

Although nested PCR (which may provide a high degree of assay sensitivity) was not used in this study, it was envisioned that potentially, a high degree of sensitivity might be achieved by increasing the PCR cycle number of the optimised single-stage assay. This would not require the addition of extra polymerase, since the enzyme used in this study (AmpliTaq Gold, Applied Biosystems) is provided in an inactive form: This allows for partial activation of the enzyme in a pre-PCR heat step, followed by "Time Release" activation, i.e. progressive activation (at 96°C) with increasing PCR cycle numbers (see AmpliTaq Gold™ package insert). This not only contributes to an increase in assay sensitivity, but also improves the specificity of the assay by providing a 'Hot Start' to the PCR reaction. In traditional 'Hot Start' PCR, the heat labile enzyme is withheld from the reaction mix until the optimum annealing temperature is reached in the reaction tube. With AmpliTaq Gold™ this is not necessary since the enzyme is inactive prior to the initial denaturing step.

It is clear that the use of single-stage PCR significantly reduces the risk of releasing amplicons. In addition, there are general precautions that the investigator should follow to further limit the contamination risk: In order to prevent amplicon contamination of samples and reagents, gel electrophoresis should be performed under negative atmospheric pressure. Even if a separate room for RNA extraction and preparation of RT-PCR reagents is allocated, gel electrophoresis should preferably still be performed under negative atmospheric pressure, particularly if the rooms share the same air-conditioning system. Since amplicons inevitably contaminate the hands, clothing and hair of the operator,¹⁸² it is also advisable that tissue processing and RT-PCR be performed before gel electrophoresis in the course of a day. Furthermore, a separate pipette should be used exclusively for gel electrophoresis. Although these general precautions are all important with conventional PCR, they are likely to be less critical with real-time PCR (e.g. Lightcycler) since the PCR product-containing tube is never opened to analyse the result.

In addition to adhering to these precautions it is also vital (especially when RT-PCR is applied for clinical purposes) to perform an H₂O-negative control with every assay run. Of note is that this control will not allow the detection of amplicon contamination if it is the nodal tissue and RNA extraction reagent that are contaminated. This could be controlled for by performing a cDNA-negative control for each patient sample (see section 3.5.3). And since this control provides a more comprehensive contamination check, it may be considered as a replacement for routine water-negative controls. Unfortunately, however, this control cannot exclude amplicon contamination of the reverse transcriptase enzyme. Furthermore, since it needs to be performed for every nodal sample, it would significantly increase the assay costs if this approach was used.

3.5.3 cDNA-specificity assessment

Another important point to consider when doing any type of RT-PCR analysis, is that the presence of residual DNA in the RNA sample²²⁸ may also cause false-positives if the primers used are not specific for cDNA. Such false-positives would arise in two different ways: First, poorly designed primers (e.g. both primers of a set within one exon) would not distinguish between residual genomic DNA and cDNA in a sample. Secondly, even well designed primers would not distinguish between cDNA and a potential pseudogene (see *Literature Review*, section 1.4.2.2).

In this study the single set of primers for each marker was initially selected on the basis of PCR product yield and specificity- see section 3.5.2. However, it was then very important to determine whether these selected primer sets would be able to distinguish between cDNA and residual genomic DNA. In order to do this, two approaches were taken: First, the cDNA position of each primer was examined (the necessary information to do this being obtained from the GenBank database <http://www.ncbi.nlm.nih.gov>). Secondly, a cDNA-negative control was performed for each primer set to further ensure that no genomic DNA amplification would occur.

The necessary information (retrieved from GenBank) to determine the primer positions for each marker is summarised in Table 3.1, and the positions of the primers for tyrosinase, Pmel-17, MART-1 and MAGE-3 depicted schematically in Figure 3.11, A-D respectively. Figure 3.12 depicts the cDNA positions of the chosen primers for PBGD, the internal control. The results of this examination show that there is at least one intron

between the primers for each of the markers. This is important since it ensures that, even if specific (genomic) DNA amplification were to have occurred, that these PCR products would have differed in size from those derived from the cDNA template. In the case of Pmel-17, the forward primer (>Pmel-17/b) is also intron-spanning, thus further reducing the risk of concomitant amplification of residual DNA.

The cDNA specificity of each primer set was then further confirmed by the absence of PCR product in the cDNA-negative control sample for each marker (Figure 3.13, lanes 2 for each marker). Here, the absence of a band of the same size as the positive control for each marker (lanes 1 for each marker) indicated that there are no pseudogenes present for any of the marker sequences amplified. The results of this section thus indicated that false-positive results would not arise due to the amplification of residual genomic DNA in the RNA extract. Furthermore, the absence of non-specific bands in the cDNA-negative controls, as well as the highly specific cDNA-derived product for each marker (as tested on UCT-Mel-1 RNA with the selected primer set, see section 3.5.2) confirmed two things: First, that amplification of specific DNA sequences with smaller intronic sequences between the primers did not occur. Secondly, it shows that non-specific binding of the primers to genomic DNA did not occur.

Table 3.1 Primer sets used in this study

Marker	Primer	Primer sequence from 5' to 3' end	Nucleotide position [§] Exon number	Product size (bp)	Ref.
Tyrosinase	>Htyr3 <Htyr2	GTC TTT ATG CAA TGG AAC GC AGG CAT TGT GCA TGC TGC TT	864-883 (Ex 2) 1083-1102 (Ex 3)	207	59
MART-1	>MART-1/b <MART-1/b	CAC TCT TAC ACC ACG GCT GA AGG TGA ATA AGG TGG TGG TGA	215-234 (Ex 2) 494-514 (Ex 5)	299	78
Pmel-17	>Pmel-17/b* <Pmel-17/a	TGC TGG AGA GGT GGT CAA GTG CTC CAG GTA AGT ATG AGT GAC	189-209 (Ex 2/3) 837-857 (Ex 6)	699	88
MAGE-3	>MAGE-3/a <MAGE-3/a	GAA GCC GGC CCA GGC TCG GGA GTC CTC ATA GGA TTG GCT CC	438-455 (Ex 1) 2703-2725 (Ex 3)	413	74
PBGD	>PBGD <PBGD	CTG GTA ACG GCA ATG CGG CT GCA GAT GGC TCC GAT GGT GA	32-51 (Ex 1) 350-369 (Ex 5)	339	78

* Unpublished forward primer for Pmel-17, designed by Hanekom and co-workers using *DNAMAN 5.1.0.0* (Lynnon Biosoft).

§ Nucleotide positions are based on the sequences with the following GenBank accession numbers: Y00819 for tyrosinase, U06654 for MART-1, M77348 for Pmel-17, U03735 for MAGE-3 and NM_000190 for PBGD. The exon information for tyrosinase is based on GenBank accession numbers AF237808 and AF237809, for MART-1 on U06654, for Pmel-17 on U31797, U31798, U31799, U31807 and U31808, for MAGE-3 on U03735 and for PBGD on M18799, M18800, D12722, X68018 and X04217.

Note: the nucleotide positions for MAGE-3 are according to the DNA sequence instead of the cDNA sequence

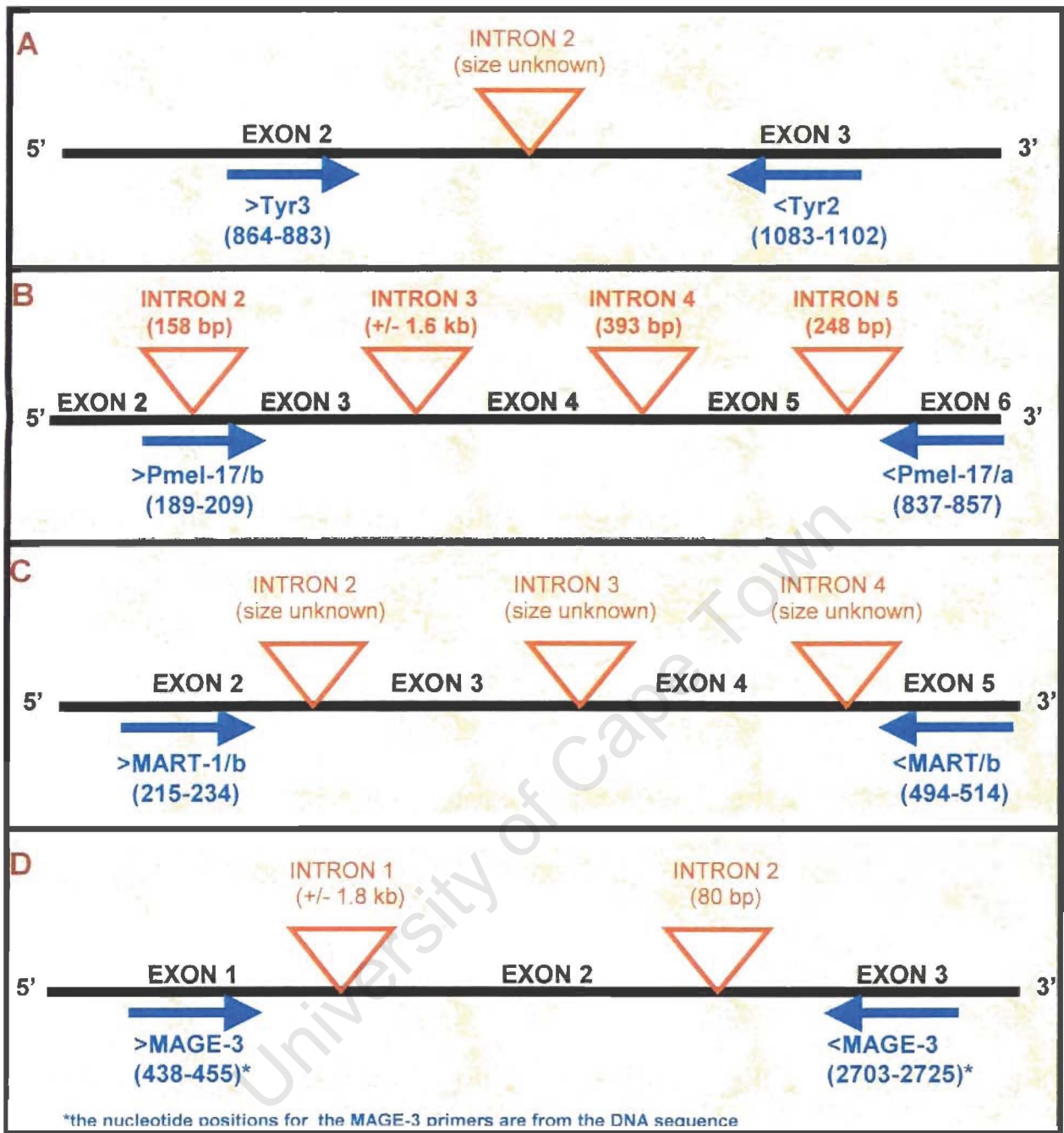


Figure 3.11. cDNA positions of the primers for tyrosinase (A); MART-1 (B); Pmel-17 (C) and MAGE-3 (D).

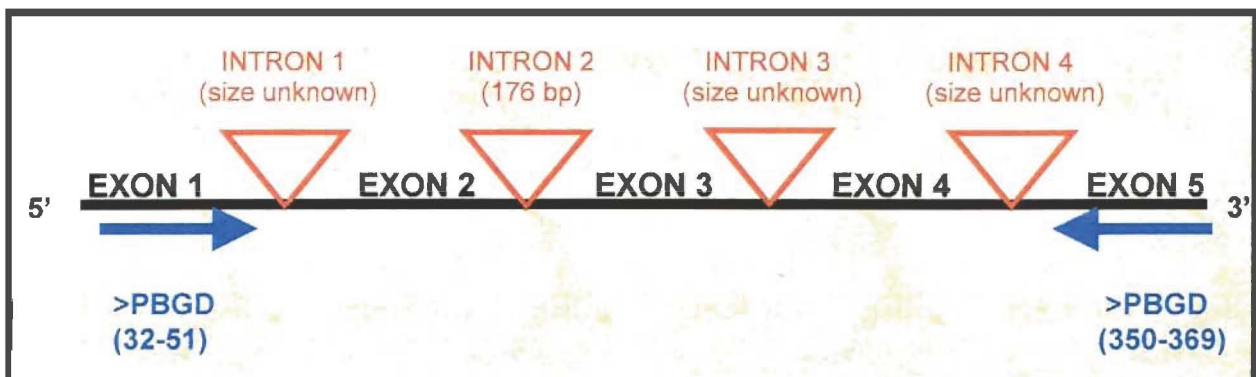


Figure 3.12. cDNA positions of the PBGD primers.

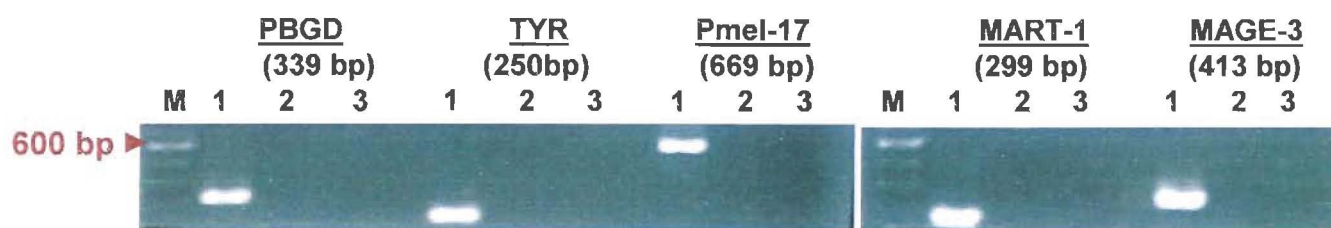


Figure 3.13. Assessment of the cDNA-specificity of each primer set. Lanes M, 100 bp molecular marker. Lanes 1, positive controls for each marker (UCT-Mel-1 RNA as RT template). Lanes 2, cDNA-negative controls for each marker (reverse transcriptase enzyme omitted from the RT-step). Lanes 3, negative (H₂O) controls for each marker. The results are representative of two independent experiments.

Ideally, when designing or selecting primers for the specific amplification of cDNA, the forward and reverse primers should be positioned such that there is a large (greater than 1 kb) intronic sequence between them. This will ensure that specific PCR amplification of genomic DNA does not occur. If there is a small intronic sequence (smaller than 1 kb and larger than 25 bp) present between the primers, this may allow some specific amplification of genomic DNA; however, such a PCR product should be easily distinguishable (on the basis of size) from the cDNA-derived PCR product. In addition and where possible, one of the two primers should be intron-spanning (as for >Pmel-17/b, Figure 3.11-B), this further reducing the risk of genomic DNA amplification.^{59, 94, 111}

Although the presence of an intronic sequence between a set of cDNA primers ensures that, even if specific DNA amplification occurs it will not lead to a false-positive result, it does not exclude the risk of a false-positive due to the presence of a pseudogene for that particular sequence. If present, a pseudogene would be co-amplified by the primers that were originally designed to amplify cDNA only, producing a PCR product indistinguishable from that derived from the cDNA template.¹⁹⁰ If a pseudogene for a particular marker is detected but a different primer set cannot be used, DNase treatment of the RT-template would help to prevent false positive results.

A tyrosinase pseudogene has been described that shares 98% homology with exons 4 and 5.²²² However, the primers used in this study to amplify tyrosinase cDNA overlap exons 2 and 3 of the tyrosinase gene⁵⁹ and should therefore not create a false positive result (confirmed in Figure 3.13). Of note is that a pseudogene homologous to the GAPDH cDNA sequence, as well as another pseudogene homologous to the β -actin cDNA sequence, have also been identified. Therefore if these housekeeping genes were

used as internal controls, a false impression of good RNA quality may lead to undetected false-negative results.^{86, 229, 230} This further strengthens the argument for the use of PBGD as a better internal control, since no corresponding pseudogene has been identified.²³¹

The above highlights the importance of performing a cDNA-negative control, at least once (at the outset of assay optimisation) for each primer set. This serves to confirm that genomic DNA amplification, particularly of a potential pseudogene will not lead to false-positives. Some investigators perform this cDNA-negative control by omitting the RT-step, i.e. performing PCR directly on RNA template. Others prefer to leave out the reverse transcriptase enzyme from the RT-step (the latter option being used in this study).

3.5.4 Optimisation of PCR cycle number to obtain maximum specificity for each marker

The specificity of a molecular marker for the detection of melanoma cells in nodal tissue relies on the premise that other cells within the node either do not express the specific marker transcripts. It is therefore of concern that all lymph nodes contain Schwann cells, these cells having been shown to express tyrosinase.^{61, 95, 112} In addition, the risk of false-positives caused by the detection of illegitimate transcripts has been used as a common argument against the use of RT-PCR for SN evaluation. These issues would seem to pose an important obstacle in the development of a molecular method for the specific detection of nodal melanoma cells. However, it is important to understand that these specific but unwanted transcripts from the background nodal cells (i.e. Schwann cell and illegitimate transcripts) are present in extremely low concentrations in nodal RNA extracts (see Literature Review, section 1.4.2.3). These transcripts should therefore only be detectable if the RT-PCR assay is highly sensitive. In fact, the detection of unwanted tyrosinase transcripts is very likely to be the cause of the apparent non-specificity of this marker in studies where investigators made use of a highly sensitive nested PCR approach.^{61, 112} In another study a low level of Pmel-17 transcription was shown to be detectable in various normal and tumour tissues/cell lines after only 35 (and even 30) PCR cycles, whilst MART-1 and tyrosinase transcripts remained undetectable in these tissues using 35 cycles.¹⁷³ What therefore really needs to be established is whether 100% specificity may still be achieved by reducing PCR cycle number?

The aim of the next set of experiments was to address this important question. By performing decreasing PCR cycles on the cDNA derived from 10 normal visceral lymph nodes, the optimal cycle number (to the closest denominator of 5 or 10) was determined for each marker. A further 10 nodes were then tested at the respective optimal cycle number to confirm specificity for each marker (see Figures 3.14 and 3.15 overleaf).

		PCR CYCLE NUMBER			
		30X	35X	40X	50X
M A R K E R	TYR		0/10	0/20	1/10*
	MAGE-3	0/10	0/20	4/10	
	MART-1	0/10	0/20	4/10	
	Pmel-17	0/20	10/10		
	PBGD	20/20			

Figure 3.14. Schematic representation of the results of the optimisation of PCR cycle number for each marker. The optimal cycle number for each marker (i.e. the maximum tested cycle number after which 0/20 normal node samples were found to have detectable marker expression) is highlighted with a green box outline.

* The figure 1/10, for example, indicates that in one of ten tested normal lymph node samples tyrosinase mRNA expression was detected at 50 PCR cycles.

The results show that both MAGE-3 and MART-1 were 100% specific in 20 nodes, at 35 PCR cycles. However, at 40 PCR cycles, unwanted MAGE-3 transcripts were detected in 4 nodes. Similarly for MART-1, unwanted transcripts were also detected in 4 nodes at 40 cycles (although these nodes were not all the same nodes as for MAGE-3). In contrast, tyrosinase was found to remain 100% specific at 40 PCR cycles or less, with unwanted transcripts being detected in 1 node at 50 PCR cycles. Pmel-17 was found to have the lowest specificity of all four markers, and 100% specificity was achieved only at 30 PCR cycles or less. At 35 cycles, unwanted Pmel-17 transcripts were detected in all of the 10 nodes tested. PBGD transcripts were detected in all 20 normal nodes even at 30 PCR cycles.

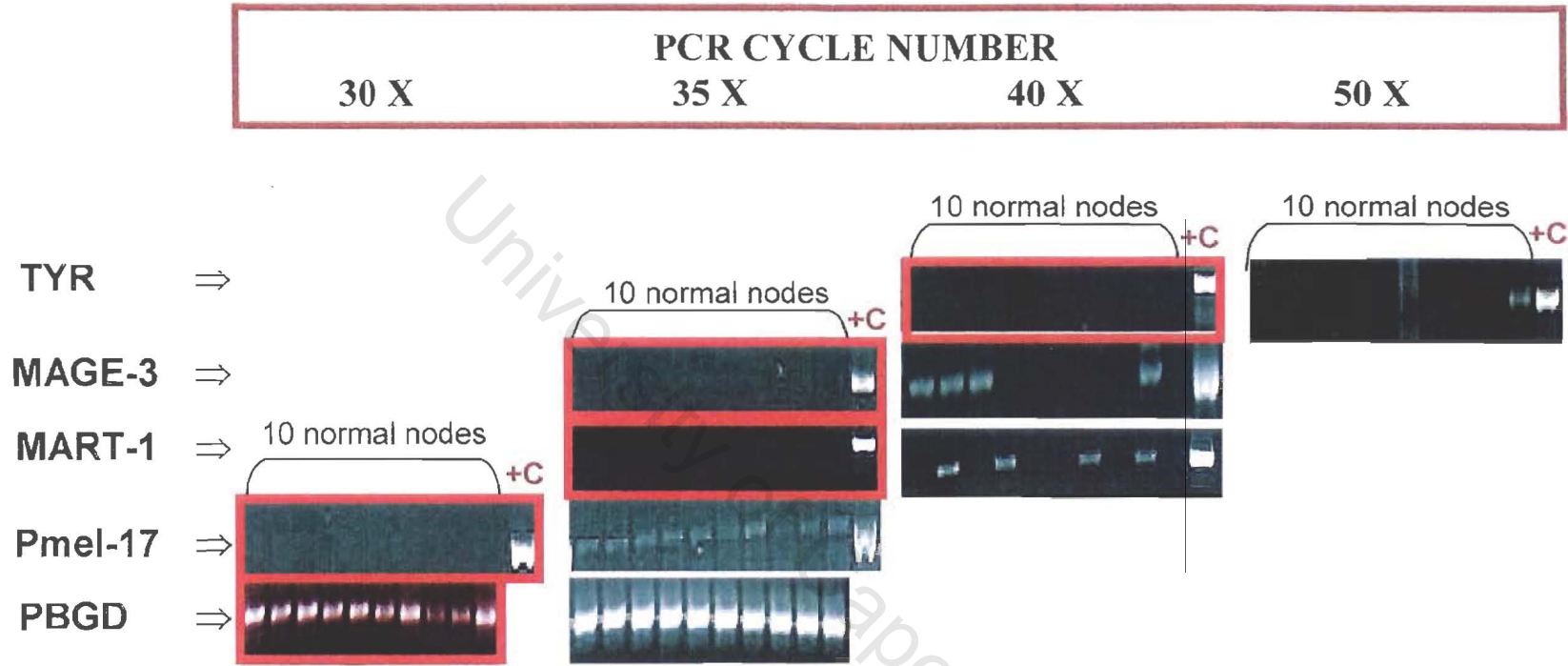


Figure 3.15. Optimisation of the PCR cycle number for each marker.

Lanes 1-10, ten normal (donor) lymph nodes. Lanes +C, positive controls (UCT-Mel-1) for each marker. The optimal PCR cycle number for tyrosinase, MAGE-3, MART-1, Pmel-17 and PBGD was 40, 35, 35, 30 and 30 respectively. Using higher PCR cycle numbers than these for each marker resulted in the detection of illegitimate transcripts. The results are representative of two independent experiments.

Therefore, in this study the following optimal PCR cycle number was chosen for each marker: 35 cycles for MAGE-3 and MART-1, 40 cycles for tyrosinase, and 30 cycles for Pmel-17. For PBGD, 30 PCR cycles was chosen as the optimal cycle number since this would provide a more sensitive assessment of RNA integrity and RT efficiency than the use of higher PCR cycle numbers. Subsequently, use of these optimised cycle numbers in this study ensured that false-positives were not caused by the detection of unwanted transcripts.

These results confirm that whilst it may seem tempting to improve the sensitivity of an RT-PCR assay by increasing the number of PCR cycles, amplification of very low levels of unwanted transcripts can become detectable if the cycle number is too high. And since in this study, unwanted transcripts of the different markers become detectable at different PCR cycle numbers, it was vital to determine the 'cut-off' PCR cycle number for each marker individually. The sensitivity of each marker was then merely dependent on this determined "cut-off" cycle number (see section 3.8).

It is vital to emphasise however that the specificity of each marker achieved using the respective "cut-off" PCR cycle number may differ when used in a different laboratory, using different reagents, and even when a different person performs the same protocol. It is worthwhile at this stage to give thought to the issue of how worldwide standardisation could be achieved should molecular evaluation of the SN be shown to be of clinical value. Ideally, every laboratory analysing SN tissue for the detection of micro-metastases should use the same RT-PCR reagents, molecular markers, PCR primers and parameters (and even the same brand of PCR machine) as well as quality controls, since this would facilitate the achievement of similar marker specificity and sensitivity. The use of an international set of control lymph node tissue RNA might then also be considered to further promote standardisation. However, each laboratory would still necessarily need to carefully determine its own safe 'cut-off' PCR cycle number for each marker i.e. the cycle number that ensures very high marker specificity whilst ensuring the highest possible sensitivity. Thereafter it would be preferable if the same person who optimised the PCR cycle number could then perform the future diagnostic PCRs.

Another crucial point of consideration when determining optimal PCR cycle number relates to the use of appropriate control tissue in order to achieve this. It is important to consider the limitations of commonly used control tissues: Markers that have been optimised for the detection of melanoma cells in the peripheral blood have not consistently been specific for melanoma cells when applied to SN evaluation.¹⁸² Likewise the abundant availability of non-melanoma tumour-involved nodes makes these attractive as control tissues for assessment of marker specificity. However, use of such control nodal tissues may significantly hamper the accurate determination of marker specificity: This is because aberrant (also referred to as "spurious") tyrosinase

expression from malignancies other than melanoma, has been reported.^{148, 188} The evidence for this is that in these studies, normal lymph nodes and nodes involved with benign disease were found not to have tyrosinase expression, whereas in the tumour-involved nodes (immunocytoma, histiocytoma, oat cell tumour¹⁸⁸ and breast carcinoma¹⁴⁸) tyrosinase expression was detectable.

Therefore, in order to establish the incidence of aberrant pigment cell-specific (PCS) marker expression in non-melanoma tumour-involved lymph nodes, 13 enlarged (tumour-involved) lymph nodes from breast cancer patients were evaluated for PCS marker expression at the optimised PCR cycle numbers. Five of these 13 nodes were found to be tyrosinase positive, four were MART-1 positive and three were Pmel-17 positive (results not shown). It can therefore be concluded that these detectable PCS markers in the breast cancer nodes are due to spurious expression. Consequently, if breast cancer-involved nodes had been used in this study to assess PCS marker specificity, it is likely that lower PCR cycle numbers would have been necessary in order to achieve specificity, or more likely, that some of these markers would have been too non-specific and therefore incorrectly regarded as being inadequate. Thus, it is vital to use appropriate control tissues (i.e. cancer-free nodes) for the necessary optimisation of PCR cycle number.

It was for this reason that normal (visceral) lymph nodes were used in this study to optimise PCR cycle number. However, it must be noted that there is still an important limitation to the use of these nodes as control tissue: This is because of the possibility that neval PCS transcripts could be detected in a proportion of sentinel nodes (SNs), which would cause a false-positive result. However, there are no "perfect" control lymph nodes (i.e. nodes with neval cells but no melanoma cells) to determine this possibility for the following reasons: Nevi appear to be absent in nodes that do not directly drain the skin (visceral nodes)¹³, and there are no available studies evaluating the presence of nevi in normal skin-draining nodes. The incidence of nevi in non-melanoma tumour draining nodes is only approximately 0.04%¹⁴⁹, and these nodes would in any case introduce other problems (as discussed above). The majority of nodal nevi are actually found in melanoma-draining SNs (approximately 4% incidence rate- see *Literature Review*, section 1.4.2.3). However, in order to serve as adequate controls, these nodes would require the absolute absence of melanoma cells; it is here that the difficulty lies, and this is discussed in section 3.6.

In conclusion, although the detection of neval cell PCS transcripts in SN cannot be excluded by optimising the PCR cycle number on available control tissues, false-positives due to the detection of illegitimate and Schwann cell transcripts have been excluded. This study also conclusively shows that nested PCR is superfluous, since unwanted marker transcripts become detectable in nodal tissues even with limited, single-stage PCR cycle numbers.

3.6 WILL THIS MULTI-MARKER ASSAY ALLOW DIFFERENTIATION BETWEEN MELANOMA AND NEVAL CELLS WITHIN A NODE?

As described above, it has been shown in this study that by optimising PCR cycle number on normal visceral lymph nodes it is possible to ensure that Schwann cell and illegitimate transcripts are not detected during analysis of the SN. It is however, still possible that false-positives may result from the detection of pigment cell-specific (PCS) transcripts that originate from nodal neval cells present in a significant percentage of melanoma-draining SNs. It was for this reason that a tumour-specific marker, namely MAGE-3 (a cancer/testis antigen) was investigated as an additional marker (see section 3.5.1).

In order to establish whether MAGE-3 would indeed be able to distinguish between neval cells and melanoma cells in a SN, the expression of this cancer/testis antigen (CTA) in these two cell types would need to be compared. Unfortunately, in this study, such a comparison was not possible, as lymph nodes containing nevi and no melanoma cells are almost impossible to obtain for the reasons discussed in section 3.5.4. Therefore, since nodal nevi are likely to be derived from dermal/epidermal melanocytes, (see *Literature Review*, section 1.4) the expression of MAGE-3 mRNA (as well as the three selected PCS markers) was determined in samples of pigmented skin, and this compared to its expression in a melanoma-involved lymph node. Of note is that, since the optimised PCR cycle number for each marker was used, no illegitimate MAGE-3 transcripts from the skin cells would be detectable.

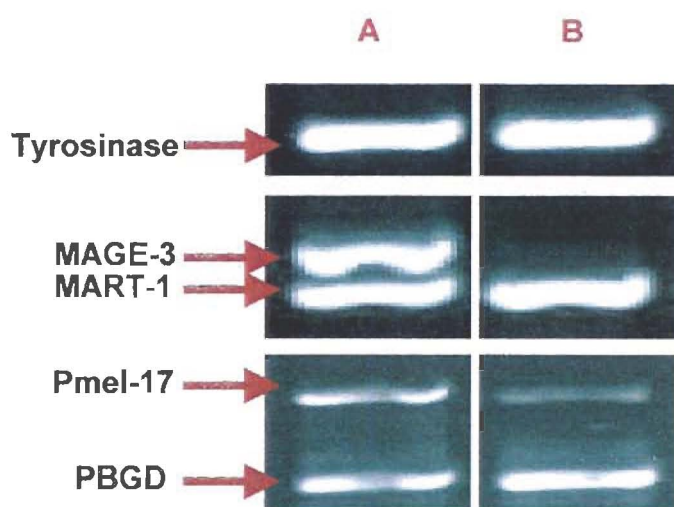


Figure 3.16. Expression of MAGE-3 and the PCS marker mRNAs in melanoma cells and skin melanocytes. Lanes A and B: Melanoma-involved node and pigmented skin respectively. The results are representative of two independent experiments.

Figure 3.16 shows that MAGE-3 was the only marker not expressed by melanocytes (Lane B), with the expression profile of the PCS markers (tyrosinase, MART-1 and Pmel-17) being identical in the melanocytes (Lane B) and the melanoma cells (Lane A). This result demonstrates that, if the PCS markers are used exclusively to evaluate the SN, it is possible that neval cells in an SN may be mistaken for the presence of melanoma cells, thus leading to a false-positive result. This is not a risk if MAGE-3 is used to detect melanoma cells, since the detection of MAGE-3 mRNA transcripts in the SN would likely be able to immediately confirm the presence of melanoma cells.

This leads to the question of why, if the PCS markers do not allow for the differentiation between neval and melanoma cells, most investigators evaluating the SN still tend to make use of PCS markers exclusively? This is most likely because the PCS markers (in particular tyrosinase) have a good detection rate for metastatic melanoma cells, and this detection rate may be further improved by using a multi-marker PCS approach (see section 3.5.1, *Rationale for the selection of markers*). In contrast, the detection rates of the currently available CTAs (including MAGE-3) for metastatic melanomas tend to be lower than those of the PCS markers. This information brings to light a concern about the value of complementing PCS marker analysis with CTAs (e.g. MAGE-3). In essence, if the MAGE-3 result for an SN is considered to be the most meaningful, then regardless of what the PCS markers reveal, these results would be ignored (because it will be assumed that the PCS transcripts are neval cell-derived). Therefore the use of MAGE-3 in combination with the PCS markers would not be any better than using MAGE-3 alone. However, the use of MAGE-3 alone would certainly lead to many false-negative results due to its sub-optimal detection rate for metastatic melanoma. One can conclude from this, that what would be ideal is the identification of a panel of tumour-specific markers (e.g. CTAs) that are shown to have a high detection rate for metastatic melanoma cells. This approach is addressed in *Future Developments*, see section 3.10.

Another possible reason for the continued use of the PCS markers is that, since the SN neval-load (like the Schwann cell-load) is very small, the neval PCS transcripts might (like the Schwann cell transcripts) remain undetected if lower PCR cycle numbers are used (see section 3.5.4). In fact, it has been found that nodal nevi have a low proliferative/metabolic rate and thus it is very likely that any neval PCS transcripts will remain undetected.

The only study (to the author's best knowledge) that has attempted to accurately determine whether neval cell-PCS mRNA transcripts are detectable in SNs by RT-PCR is a study that has made use of sections of melanoma-draining SNs that had been shown (by histological examination of other sections) to contain nevi and to be free of metastases.¹²⁸ These investigators found that two of eight such SNs were positive for their optimised tyrosinase assay. It is likely that the lack of other studies addressing the risk of false-positives due to nodal nevi is due to certain limitations associated with the necessary experimental approach itself (as used above): First, acquisition of these lymph nodes is extremely difficult, as only 8.5% of SNs that are free of melanoma metastases contain nevi. Secondly, identification of suitable nodes will require intense examination of the SN in order to exclude the presence of melanoma deposits, whilst confirming the presence of nodal nevi; this is very labour intensive and costly. Thirdly, different sections of the SN are necessarily examined by histopathology and RT-PCR; thus if any of such SNs were shown to be RT-PCR positive for any of the PCS markers it still would not absolutely confirm that the presence of nodal nevi would cause a false-positive result. This is because the co-existence of metastatic deposits in the section of node evaluated by RT-PCR cannot be excluded in these control nodes, and these histologically undetected malignant cells (rather than nevi) could be producing the detectable PCS transcripts. Therefore the development of adequate controls to ensure that PCS markers will not cause any false-positives is not possible at present.

In conclusion, it has been shown in this study that the use of a CTA offers a means of reducing, and even eliminating, the risk of false-positives due to the presence of SN nevi. However, it must be noted that this approach currently has the significant limitation of increasing the risk of false-negatives due to a probable reduction in the detection rate.

3.7 ASSESSMENT OF THE DETECTION RATE OF EACH MARKER

Heterogeneity of marker expression occurs amongst tumour cells, particular at later stages of tumour progression.^{74, 85-87} Therefore, in theory the use of a panel of molecular markers should improve the detection rate for metastatic melanoma deposits. In order to determine whether this was indeed the case, the expression of each marker in eight melanoma cell lines, as well as in four melanoma-involved lymph nodes (all enlarged), was determined (using the optimised PCR cycle number for each marker) and the detection rates listed in Figure 3.17. (Note: the "detection rate" of a marker is defined as

the number of samples that are positive for a marker in a population of samples. It should always be kept in mind when considering such results, that detection rate is also dependent on the sensitivity of the assay.)

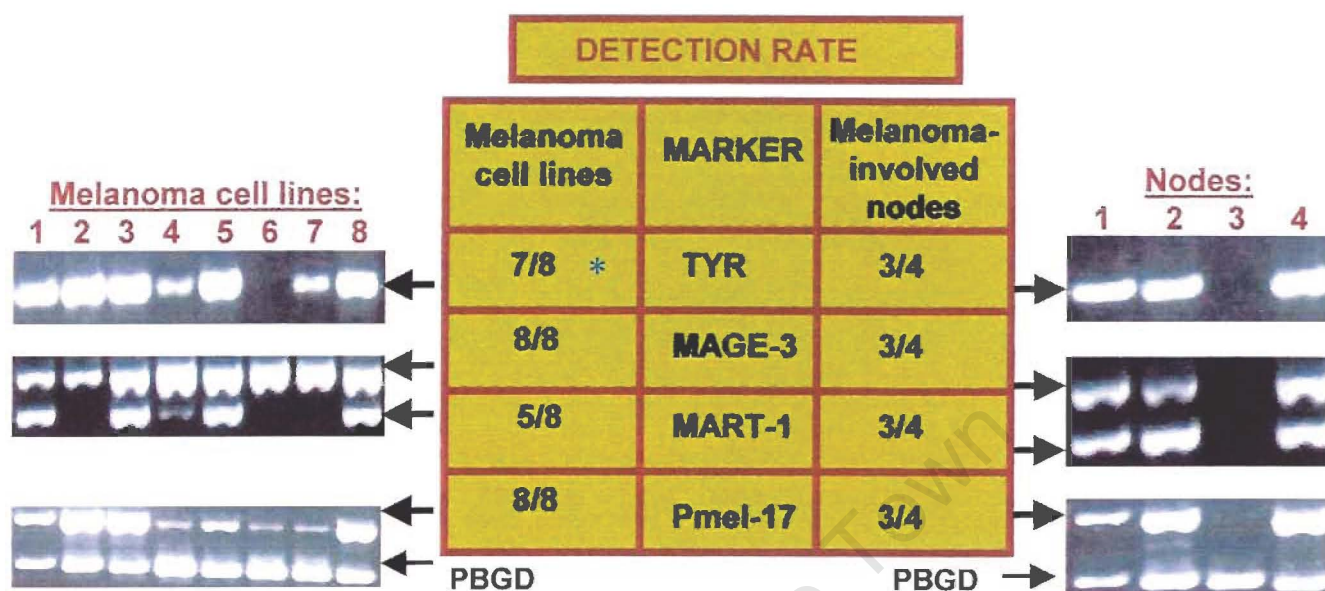


Figure 3.17 Assessment of the *in vitro* and *in vivo* detection rates of each marker. To the left of the box: UCT-Mel-1 to -8 cell lines. To the right of the box: Melanoma-involved lymph node number 1 to 4. MART-1 plus MAGE-3, and Pmel-17 plus PBGD were co-amplified. The results are representative of two independent experiments.

* Detection rate: The figure 7/8 indicates, for example, that seven of the eight melanoma cell lines tested had detectable tyrosinase mRNA expression at 40 PCR cycles).

The results of this study show that for the eight melanoma cell lines tested, both MAGE-3 and Pmel-17 had a 100% detection rate (Figure 3.17, lanes 1-8 on the left). Only MART-1, and to a lesser degree tyrosinase, showed lower detection rates amongst the cell lines. Of the four lymph nodes tested, the results show that nodes 1, 2 and 4 expressed all four markers (Figure 3.16, lanes 1, 2 and 4 on the right). Node number 3, however, did not express MAGE-3 or any of the PCS markers (except for a very faint Pmel-17 band not visible on print). Overall, these results show that the multi-marker approach used in this study did not improve the assays detection rate for melanoma cell lines or melanoma-involved nodes, since a single-marker assay with either MAGE-3 or Pmel-17 provided as high a detection rate as the multi-marker assay did. However, since only a small number of cell lines and lymph nodes were tested, this conclusion would have to be validated by determining the expression profiles of many more tissue samples.

Of note is that the relative lack of marker heterogeneity found amongst the melanoma cell lines is in accordance with reports that melanoma cell lines, like melanocytes, tend to lack marker heterogeneity.⁸⁷ This is to be compared with the high degree of marker heterogeneity reported in metastatic melanoma lesions (see above). Thus, the use of melanoma cell lines, as done in this study and by other investigators^{74, 87, 88, 95} to determine the detection rate, does not in fact accurately reflect the *in vivo* detection rate of the markers for nodal melanoma metastases. As for the cell lines, the use of primary melanoma tissues (as done in some studies^{87, 88, 95, 128}) would also not provide an accurate indication of the *in vivo* detection rate of a marker, since heterogeneity of marker expression is less evident in the early stages of tumour progression.⁸⁵

Although the number of melanoma-involved nodes (all confirmed positive by H&E examination) available for this study was limited, it was interesting that lymph nodes 1, 2 and 4 showed lack of heterogeneity in marker expression. This result differs from other reports in which significant heterogeneity was found in metastatic deposits.^{74, 85-87} Clearly more nodes will need to be examined to resolve this. Another point of note is that node 3, which expressed none of the markers (except for a very faint Pmel-17 band), was one of two lymph nodes that contained unpigmented melanoma cells (compared to nodes number 1 and 2 which contained metastatic deposits with at least focal areas of pigmentation/hyperchromasia). The lack of marker expression in many amelanotic nodal metastases indicates the need for a marker(s) that is/are capable of more accurately detecting amelanotic melanoma metastases.⁸⁷ The CTAs are good candidates since their expression seems to be unrelated to the pigmentation pathway. Unfortunately, MAGE-3 also was not detected in node 3; however, there are other members of the MAGE-A family (e.g. MAGE-6 and MAGE-12) that have recently been shown to have a better detection rate for melanoma than MAGE-3, and these might therefore be more useful.²²⁷

Lastly, it is important to note that the nodes used in this, and many other studies (i.e. nodes involved by macroscopic melanoma deposits) might not be the ideal tissue to accurately evaluate the detection rate of the markers for microscopic nodal metastases. This is because markers with a low sensitivity (e.g. Pmel-17- see section 3.8) might appear to provide a high detection rate when analysing nodes containing macro-metastases, but would actually have a low detection rate if nodes containing micro-metastases were analysed. Moreover, it has been shown that the marker expression

profile of nodal micro-metastases differs from that of macro-metastases, with the former proposed to be in a “dormant” state.^{111, 208-210} (In this “dormant” state, cells might be quiescent and might therefore not express all the markers that fully metastatic cells express.) Therefore, ideally, multiple SNs containing micro-metastatic deposits (confirmed by histopathology) should be used if an accurate assessment of the *in vivo* detection rate of the markers is to be achieved.

3.8 ASSESSMENT OF THE SENSITIVITY OF EACH MARKER

Once assay specificity had been achieved by optimising PCR cycle number (see section 3.5.4), an assessment of the assay’s sensitivity could be determined without the risk of detecting illegitimate or Schwann cell mRNA transcripts. To the investigator’s best knowledge, all studies using RT-PCR for the detection of melanoma cells in lymph nodes assess the sensitivity of their assay by spiking decreasing numbers of melanoma cells into a fixed number of lymphocytes.^{75, 112, 148, 151, 158} In this study, the sensitivity of each marker was assessed with a more accurate approach, i.e. by spiking decreasing numbers of melanoma cells into equivalent amounts of normal nodal tissue.

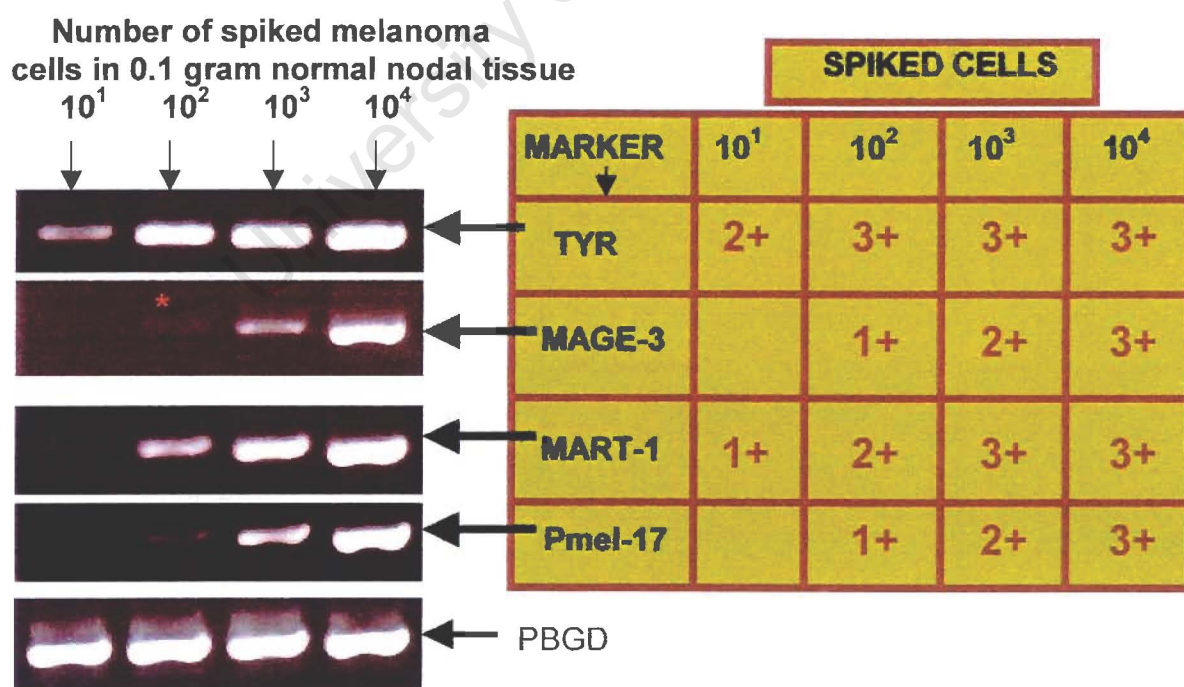


Figure 3.18. Assessment of the sensitivity of each marker by using normal nodal tissue spiked with decreasing numbers of UCT-Mel-1 cells. 1+, 2+ and 3+ indicate the intensity of the PCR product bands, 1+ being very faint and 3+ being very strong. The results are representative of two independent experiments.

* Note, MAGE-3 band for 10² melanoma cells not visible on print, but visible on original.

The results (Figure 3.18) show tyrosinase to be the most sensitive of the four markers, allowing the detection of as few as 10 UCT-Mel-1 cells in a background of 0.1 g normal nodal tissue. MART-1 is almost as sensitive (100 melanoma cells in 0.1 g of normal nodal tissue), with MAGE-3 and Pmel-17 enabling the confident detection of 1000 melanoma cells in 0.1 g of normal nodal tissue (although the 100 melanoma cell dilution was also weakly detectable for both markers). Overall, good sensitivities were achieved for all the markers, even with the reduced PCR cycle numbers needed to achieve 100% specificity.

Based on the fact that there are about 2×10^8 human diploid fibroblasts (16-18 μm in diameter) per gram of wet weight²³², it may be estimated that the sensitivity achieved for tyrosinase in this study (with 10 melanoma cells detected in 0.1 g of normal nodal tissue) was 1 melanoma cell in 2×10^6 background cells. This compares well with reported and generally accepted RT-PCR sensitivities of 1 cancerous cell being detectable in a background of 10^6 - 10^7 normal cells.^{75, 112, 146, 151, 158} In comparison, H&E staining and immunohistochemical analyses are typically capable of detecting one cancerous cell in a background of 10^4 and 10^5 normal cells respectively.¹⁴⁶ Thus, for tyrosinase, it can be calculated that molecular analysis is 200-times more sensitive than H&E staining and 20-times more sensitive than standard IHC.

It should be noted that the sensitivity assessment is based on the expression of these markers in only one cell line, UCT-Mel-1, and is likely to be different for other cell lines. Furthermore, the expression of markers is often up-regulated, and even induced, in cultured cells,^{87, 111} and therefore this *in vitro* sensitivity assessment is not necessarily representative of the *in vivo* scenario. It would therefore be more appropriate to spike melanoma cells derived from several different melanoma-involved lymph nodes into normal nodal tissue to gain a more accurate assessment of marker sensitivity. However, there are difficulties with this experimental approach: First, the isolation of viable tumour cells from the nodal tissue would be technically difficult, and secondly, it would require the use of a non-selective melanoma immunohistochemical marker that will ensure 100% detection of the heterogeneous cancer cell population. Even this approach will not provide an absolutely accurate assessment of the sensitivity of the assay for micro-metastatic deposits, as these cells may vary in their expression levels when compared to cells derived from macroscopic metastases (see section 3.7). However, it is likely that such an accurate determination of assay sensitivity is unnecessary. Rather, it will

probably be much more important to correlate PCR product yield (reflecting tumour burden) with clinical outcome.

3.9 REPRODUCIBILITY

One of the problems with the development of a molecular assay for the detection of a certain cell type amongst background cells is that the risk of false-negatives is significantly increased when there are low numbers of specific mRNA template molecules (as might be the case with melanoma nodal micro-metastases). This is because even minor variations in RNA extraction and RT-PCR efficiency, and/or minor degrees of RNA degradation may result in false-negatives, since these minor variations usually go undetected by the conventional internal control markers (i.e. housekeeping genes). The RT step especially has been identified as the main source of RT-PCR variability, with the RT enzyme being sensitive to salts, alcohols, phenol or protein contaminants that may remain from the RNA extraction step.^{89, 111, 184} In addition, a form of sampling error may result in false-negatives, whereby the specific RNA aliquot evaluated may not always contain sufficient mRNA copies to produce a positive result when there are very low numbers of amplifiable mRNA targets present in the tissue being analysed.⁷⁸ Therefore in order to limit the occurrence of sporadic false-negatives, due to the above-mentioned variations in the protocol, it is essential that a high degree of reproducibility for the entire protocol be attained in each laboratory.^{65, 66, 86, 90, 103, 104, 182} This does not however, replace the need to perform a negative (H₂O) with each RT-PCR run.

In order to obtain an accurate assessment of the reproducibility of the nodal processing protocol as well as the RT-PCR assay used in this study, eight nodal tissue samples (0.1g each) were each spiked with 10 UCT-Mel-1 cells. This was done essentially as described in *Materials and Methods* (section 2.14), except that the melanoma cell lysate was also subjected to the nodal homogenisation process. PCR product yield for tyrosinase and PBGD was identical in the eight nodal samples, with the results from four of these samples shown in Figure 3.19 (lanes 1-4). These results suggest that the entire protocol used in this study had a good degree of reproducibility.

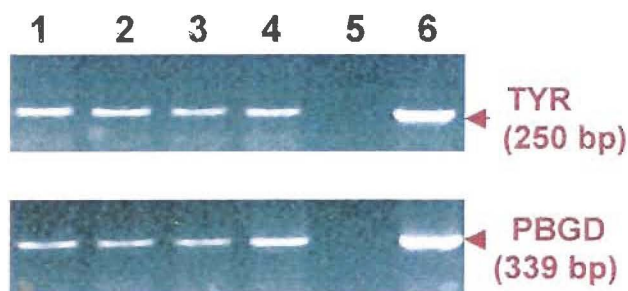


Figure 3.18. Assessment of the reproducibility of the protocol used in this study. Lanes 1–4, tyrosinase and PBGD yields from four independent nodal tissue samples each spiked with 10 UCT-Mel-1 cells and subjected to the entire protocol (i.e. nodal processing and RT-PCR). Lane 5, negative (H_2O) controls for tyrosinase and PBGD. Lane 6, positive controls for tyrosinase and PBGD (UCT-Mel-1 RNA as RT template). The results are representative of two independent experiments (i.e. a total of eight nodal samples).

Reproducibility of a protocol will, in particular, provide a means to assess operator consistency. Subsequent to achieving reproducibility, it is preferable that the same reagents, PCR machine, protocol and even operator be used for each assay in order to maintain this reproducibility. Finally, a higher false-negative rate is likely to occur in those laboratories with lower assay sensitivities. Therefore, ideally, every laboratory analysing SN tissue for micro-metastases should use the same reagents, protocol (particularly one that minimises RNA degradation and achieves maximum RT-PCR efficiency) and quality controls, since this would facilitate the achievement of similar sensitivities.

3.10 CONCLUSIONS AND FUTURE DEVELOPMENTS

The use of RT-PCR to detect melanoma nodal micro-metastases is potentially an extremely cost-effective and sensitive alternative to the histopathological evaluation of the sentinel node (SN) in primary melanoma patients. However, the main obstacle preventing this technique from becoming clinically applicable is its apparent lack of specificity. The main aim of this study was therefore to investigate whether it would be possible to develop a highly specific RT-PCR assay for the detection of small numbers of melanoma cells in nodal tissue. Other important aspects that were particularly addressed in this study were sensitivity, reproducibility and improved detection rate of the molecular assay.

In order to achieve the above, RNA from various melanoma cell lines as well as nodal tissues (normal visceral and melanoma-involved nodes) were used to optimise the protocol to be used in this study. An efficient and practical tissue homogenisation method was developed to improve reproducibility and limit the risk of false-negatives due to RNA degradation and RT-inefficiency. The use of a sensitive internal control marker (i.e. PBGD) was shown to be able to further limit such causes of false-negatives. The decision to use a multi-marker approach was taken in order to try to improve the detection rate of the tyrosinase single-marker RT-PCR assay. Although this approach did not appear to enhance detection rate, it is still too early to make any definite conclusions, and a larger number of tissues will need to be evaluated. The specificity of the PCR primers selected in this study was confirmed, and the use of single-stage versus 2-stage (nested) PCR all contributed to reducing the risks of false-positives. Normal visceral lymph nodes were used to optimise PCR cycle number in order to exclude the detection of unwanted transcripts. Good assay sensitivity and reproducibility were achieved and the necessary positive and negative controls were performed with each batch of samples to exclude sporadic false-positives and -negatives.

In conclusion it was shown in this study that it is possible to develop a RT-PCR assay that is highly specific and sensitive for the detection of melanoma cells in normal visceral lymph nodes. However, it is still possible that false-positives may be caused by the exclusive use of the pigment cell-specific (PCS) markers to evaluate a SN. This is because of the presence of nodal nevi in a significant proportion (8.5%) of melanoma-draining SNs that are metastasis-free, these nodes being almost impossible to obtain as controls.¹³⁰ It was for this reason that, in this study, a tumour-specific marker, namely MAGE-3 (a cancer/testis antigen, CTA), was tested and confirmed to allow differentiation between melanoma cells and skin melanocytes (representative of nodal nevi). Unfortunately, this approach of combining three PCS markers with one CTA has a significant limitation: Melanomas that do not express MAGE-3 will produce false-negative results, since any melanoma cell-derived PCS transcripts will be assumed to come from neval cells in the SN. In light of this, it is recommended that more emphasis be placed on the development of a panel of CTAs, or other tumour-specific markers, that have the same or better detection rate as the currently available PCS markers for melanoma micro-metastases. The development of such a panel is furthermore, likely to be useful in the detection of metastatic disease in patients with other carcinomas; this is because it has been shown that, just as nodal neval cells are detected in some melanoma-draining

SNs, epithelial inclusion cells are detected in some of the draining nodes of many other tumour types (e.g. breast epithelial cells in breast cancer).¹⁹⁴ Thus the use of tissue-specific epithelial markers for the detection of the respective metastases in SNs may carry a similar risk of false-positive SN result.¹¹¹

The MAGE-A, GAGE, BAGE and XAGE genes are all CTAs that code for distinct antigens recognised by autologous cytolytic T lymphocytes, and are thus being investigated as promising targets for active specific tumour immunotherapy.^{227, 233-236} Many malignancies have been evaluated for MAGE-A, GAGE, BAGE and XAGE mRNA expression, including melanoma,^{178, 186, 227} breast cancer,¹⁸⁶ eosophageal cancer,²³⁴ gastric cancer,^{234, 236} colon cancer²³⁷ and rhabdomyosarcomas.²³³ Thus far, the MAGE-A genes have generally been shown to have the best detection rates. Unfortunately individually, the MAGE-A genes have a low frequency of expression. Therefore, one group has developed a unique assay using 'universal' MAGE-A primers that simultaneously amplify several MAGE-A genes.¹⁸⁶ Due to better technologies like differential display, novel CTAs (e.g. TRAG-3, RBP1L1 and MDA-7) are being discovered at an increasing rate, thus providing more possibilities for optimal detection rate.²³⁸⁻²⁴⁰

Another potential means of detecting metastatic tumour cells in RLNs is to evaluate for microsatellite instability. These genetic errors are merely a reflection of the genetic instability of tumour cells.²⁴¹ Up to 25% of melanoma patients demonstrate microsatellite instability of their primary tumours at one of 10 loci examined.²⁴² Microsatellite instability is found more frequently in metastatic melanoma lesions than the corresponding primary tumour.²⁴³ Since these DNA mutations are identified by PCR (without the need for RT) these markers could serve as attractive alternatives, or at least be used to complement RT-PCR evaluation of nodal tissue. Furthermore, the detection of free-circulating DNA microsatellites in the blood of melanoma patients could provide a potentially less invasive route of obtaining a surrogate molecular marker of sub-clinical disease progression.²⁴⁴

Once an appropriate panel of CTAs, or other tumour-specific molecular markers, has been selected on the basis of specificity (by evaluation of at least 50 negative control nodes and skin melanocytes), these markers would then have to be evaluated on SN tissues: An accurate assessment of marker detection rate would be possible by evaluating approximately 20-30 SNs containing histologically-confirmed melanoma

micro-metastases. Once such a panel has been shown to provide specific and optimum detection of melanoma SN micro-metastases, the PCS markers could be excluded to ensure a zero false-positive rate. In addition, the CTAs are more likely to have prognostic value than the PCS markers, since the former are essentially involved in the metastatic process while the latter are involved in the pigmentation pathway. Thus, in conclusion, the potential causes of false-positives and false-negatives should not be used as reasons to exclude RT-PCR for SN evaluation, but rather viewed as surmountable challenges in optimising a valuable tool for clinical application.

Assuming that all the technical problems (discussed above) are overcome, the next step would be to determine whether the optimised RT-PCR assay will provide an accurate assessment of the presence of clinically-significant nodal metastases. This would require long-term follow-up of patients who have undergone SN biopsy, and whose node(s) has been analysed by both RT-PCR and histopathology. Importantly, it must be kept in mind that 'submicroscopic' metastases are probably more likely to be restricted to the SN, and therefore that patients who are IHC-negative/RT-PCR-positive may often be cured by the SL procedure itself. This would give the false impression that RT-PCR has a high false-negative rate. Here lies the tremendous advantage of 'real time' PCR: Semi-quantitative analysis of tumour burden may be found to correlate with disease progression and in this way a 'cut-off' PCR sensitivity could be determined to limit detection to just the clinically-significant nodal metastases. Moreover, 'real time' PCR would significantly reduce the speed of the protocol and also facilitate multiplex PCR, thus introducing further cost-savings.

Finally, the establishment of a dynamic multidisciplinary team, with each discipline continuously informed regarding the progress and limitations of the other discipline, is essential to guide the protocol development in a clinically significant direction.

APPENDIX: Methods and solutions

A.1 Laboratory precautions

Whenever working with human tissues, latex gloves (powder washed off) were worn, and universal safety precautions were adhered to. All sharps (including glassware and blades) were disposed of in a 'sharps' container, and together with all disposables used, sent for incineration.

All laboratory plastic and glassware used was either new, or treated to degrade RNases: DEPC-treated (0,01%), or baked at 180°C for 8 hours if glassware. All reagents that were used were of 'AnalaR' quality, and all ddH₂O used was DEPC-treated (see A.17). Only certified RNase-/DNase-free centrifuge tubes (Promega, Madison, USA) were used and these were further gas sterilised (using gasolene oxide). Only aerosol-resistant-tips (pre-packed) (Promega) were used when dispensing the enzymes. Gloves were generally worn throughout the protocol, and further precautions were taken not to touch the insides of lids.

The node preparation/dissection, RNA extraction, and RT and PCR reactions were all performed in a separate room from where gel electrophoresis was performed. Tubes containing PCR products were opened and gel electrophoresis was always performed under negative atmospheric pressure to minimize subsequent amplicon contamination of nodal/RNA samples or RT-PCR reagents.

A.2 Instrument cleaning and sterilisation

The pair of dissecting forceps and the scalpel handle used to dissect the lymph nodes were treated in a very specific way after each nodal dissection. The instruments were first washed with tap water and soap to remove any blood and/or obvious pieces of tissue attached. They were then placed in 100% Jik (containing 3,5% m/v Sodium Hypochlorite active ingredient) for 15-30 mins, rinsed with tap water and then rinsed with DEPC-treated ddH₂O and autoclaved.

A.3 Preparation of the stainless-steel balls

The balls were soaked in 100% ethanol for 15 mins, then rinsed in DEPC-treated ddH₂O and dried at 37°C. The balls were stored in a sterile, RNase-free container until used.

A.4 Steel versus stainless-steel balls for the nodal homogenisation

Steel balls were initially used to homogenise the nodal tissue. However, it was noted that these balls appeared to be causing a chemical reaction with the RNA isolation reagent, since the resultant aqueous phase (containing the τ RNA was discoloured when subsequently compared to that homogenised using a stainless-steel ball). Also, the mRNA extraction from nodal tissue consistently failed to yield a RT-PCR product, even though the same τ RNA produced a good product when the steel balls were used. The mRNA from cell lines produced good product, confirming that there was nothing wrong with the mRNA extraction protocol.

A.5 RNA extraction from cell cultures

A commercially available RNA isolation reagent, namely TriPure Isolation Reagent (Boehringer Mannheim, BM), was chosen as it was felt that the long-term advantage of using a standardised reagent would improve consistency of results and ease of applying the protocol. As per the package insert, 1 ml of cold TriPure Isolation Reagent (4°C) was added to 5-10 x 10⁶ cells. Complete cell lysis was achieved by repetitive pipetting until the sample appeared homogenous, this then being transferred to a 2 ml polypropylene microfuge tube. The cell lysate (in Tri-Pure) was incubated at room temperature for 5 mins to ensure the complete dissociation of nucleoprotein complexes. Chloroform (0.2 ml per 1 ml TriPure required) was added and the tube vortexed vigorously for 15 seconds. The tube was again incubated at room temperature for 5-10 mins and then centrifuged at 12 000 g for 15 mins (at 4°C) to separate the solution into three phases. Of the colourless upper aqueous phase, 350-500 μ l were carefully transferred to a new 1.5 ml microfuge tube. This was done without disturbing the interphase, and leaving behind a thin layer of the aqueous phase. The interphase (containing mainly DNA) and lower red organic phase (containing mainly protein) were then discarded appropriately. Then 500 μ l isopropanol (at 4°C) was added for each 1 ml of TriPure Reagent initially used. The sample was mixed by inversion and stored overnight (or for a few days) at -20°C to allow for RNA precipitation.

On resuming the RNA extraction, the sample was centrifuged at 12 000 g for 10 mins at 4°C. The supernatant was carefully poured off from the pellet and discarded. Then 1 ml 80% ethanol (at room temperature) was added to the RNA pellet, washing the pellet by briefly vortexing, and then centrifuging at 7500 g for 5 mins at room temperature. The supernatant was poured off, with the excess ethanol being carefully pipetted off the pellet

if necessary. The RNA pellet was resuspended in 100 μl of DEPC-treated ddH₂O by repetitive pipetting until completely dissolved. The sample was aliquoted into 5 μl amounts in 500 μl microfuge tubes and stored at -75°C until needed for RT-PCR.

An aliquot of the RNA was diluted 1 in 100 with DEPC-treated ddH₂O in order to determine RNA concentration and purity by spectrophotometry.

OD_{260/280} ratio of 1.8 – 2.0 for pure RNA

Ratio < 1.8 = protein contamination

Ratio > 2.0 = phenol contamination

The RNA concentration ($\mu\text{g}/\mu\text{l}$) was calculated as = $\frac{\text{OD}_{260} \times 100}{\text{dilution factor}}$

25

A.6 mRNA extraction

For each mRNA extraction, 40 μg of τRNA (in 100 μl DEPC-treated ddH₂O) was heated at 90°C for 2 min and then snap-cooled on ice. Then 200 μl of TEN₁₀₀ binding buffer (BM) was added to the denatured τRNA . This was mixed vigorously, and then 50 μmol of biotin-labeled oligo(dT)₂₀ probe (BM) added and mixed vigorously. This was incubated at 37°C for 5 min, and then 0.33 mg of washed* Streptavidin magnetic particles (BM) added and mixed vigorously. The mix was incubated again at 37°C for 5 min. This was then subjected to magnetic separation on a magnetic particle separator (BM) for 1 min. The supernatant was pipetted off and the magnetic particles resuspended in an appropriate volume of TEN₁₀₀₀ washing buffer (BM). The magnetic separation and resuspension was repeated twice, resuspending the magnetic particles in 10-20 μl of DEPC-treated ddH₂O after the third separation. The resuspension was heated to 65°C for 2 min, this eluting the mRNA off from the magnetic particles, which were then removed using the separator and transferring the supernatant to a fresh microfuge tube. This mRNA extract was then kept on ice until used for the RT reaction, as for τRNA .

*Per mRNA reaction: 0.33 mg (33 μl) of the commercially-available beads were washed twice in TEN₁₀₀ and then resuspended in 10 μl of TEN₁₀₀.

A.7 The OD conversion formula: $\frac{\text{OD/ml} \times 1000}{10 \times (-\text{mer})} = \mu\text{M (or pmoles}/\mu\text{l)}$

A.8 RT-mix (n=1)

ddH ₂ O	/
10x buffer	2.0 μl
25 mM MgCl ₂	4.0 μl
10 mM dNTP's	2.0 μl
50 μM oligo(dT) ₂₀	1.0 μl
50 U/ μl RNase inhibitor	0.5 μl
20 U/ μl MuLV enzyme	<u>0.5 μl</u>
	10.0 μl

A.9 PCR-mix (n=1)

	<u>SINGLE-MARKER</u>	<u>MULTIPLEX (2 markers)</u>
ddH ₂ O	13.6 μl	11.8 μl
10x buffer	2.5 μl	2.5 μl
25 mM MgCl ₂	1.5 μl	1.5 μl
>primer(s)	1.0 μl	2.0 μl (1 μl of each >primer)
<primer(s)	1.0 μl	2.0 μl (1 μl of each <primer)
5 IU/ μl Taq Gold	<u>0.4 μl</u>	<u>0.4 μl</u>
	20.2 μl	20.2 μl

A.10 Agarose-gel electrophoresis

The frozen PCR products were allowed to thaw at room temperature. To each cold 25 μl sample, 6 μl of a 5x agarose gel-loading buffer was added. Ten μl of each sample was loaded in each lane of the 2% agarose mini-gel, immersed in 0.5x TBE-buffer. Eight μl of a 100 bp DNA ladder (GibcoBRL) was loaded alongside the products. The products were electrophoresed at 80 volts/cm for approximately 45 mins. The images were visualised and captured using a UV doc system (UVIttec Limited, Cambridge, UK).

A.11 RPMI 1640 tissue culture medium for routine culturing of cells (RP5)

52 g RPMI 1640 powder medium (Sigma) in 4000 ml ddH₂O.

10.0 g NaHCO₃

2.5 x 10⁵ Units penicillin (50 U/ml) (Novo Nordisk)

0.1 g streptomycin sulphate (20 µg/ml) (GibcoBRL)

Adjust pH to 7.1 with 1M HCl

Make up to 5000 ml with ddH₂O

Sterilise the solution through a 0.2 µm filter (pH should be approximately 7.4)

Store in 500 ml aliquots at 4°C

Supplement with 5% fetal calf serum (FCS) when ready to use each aliquot.

If aliquot has been reconstituted more than 1 month before use, add 2 mM L-glutamine

A.12 L-glutamine (200 mM)

3.0 g L-glutamine powder (146.15 M) in 100 ml ddH₂O

Store in 10 ml aliquots at -20°C

A.13 RPMI 1640 storage medium for cells (RP10)

As for culture medium above, but with the addition of 10% FCS instead of 5%, plus 10% dimethylsulphoxide (DMSO)

A.14 Fetal calf serum (FCS)

Heat the bottle of FCS (Highveld Biological) once at 56°C for 30 mins to inactivate complement factors and pre-filter (Millipore type AP)

Store in 50 ml aliquots at -20°C

A.15 10x Phosphate-buffered saline (PBS)

80.0 g NaCl (1.37 M)

2.0 g KCl (0.03 M)

14.4 g Na₂HPO₄ (0.08 M)

2.4 g KH₂PO₄ (0.01 M)

Make up to 1000 ml with ddH₂O and sterilise through a 0.2 µm filter. Store at room temperature. Dilute 1 in 10 as a working solution (the pH should be approximately 7.4)

A.16 10x Versene-buffer

40.0 g NaCl (1.37 M)

1.3 g Na₂EDTA

1.41.0 g KCl (0.03 M)

5.7 g Na₂HPO₄·2H₂O (0.08 M)

1.0 g KH₂PO₄ (0.01 M)

Make up to 500 ml with ddH₂O

Sterilise the solution through a 0.2 µm filter and store at room temperature

Dilute 1 in 10 as a working solution (the pH should be approximately 7.4)

A.17 DEPC-treated ddH₂O

0.01% diethylpyrocarbonate (DEPC) in ddH₂O (in a fume hood)

Shake vigorously, and incubate for 2 hours or overnight at 37°C

Autoclave to inactivate the residual DEPC

A.18 2% Agarose mini-gel

Melt 1 g of agarose powder in 50 ml 0.5 x TBE buffer (microwave)

Add 4 µl ethidium bromide (10 mg/ml) for a final concentration of 0.8 µg/ml

Pour into mould containing comb and allow to set

Run gel in 0.5x TBE buffer

A.19 10x TBE buffer

216 g Tris (0.89 M)

110 g Boric acid (0.89 M)

80 ml EDTA (0.02 M) Make up to 2 l with DEPC-treated ddH₂O

A.20 6x Agarose gel-loading buffer (6x AGLB)

Dissolve 0.025 g bromophenol blue (0.25%) and 0.025 g xylene cyanol FF (0.25%) in 7 ml ddH₂O.

Add 3 ml glycerol (30%) and store in 1 ml aliquots at 4°C

Add 5 µl 6x AGLB to 25 µl PCR product and load on agarose gel.

Electrophorese at 50 Volt/cm until dye completely entered into gel from well, then increase to 80-100 Volt/cm.

A21 Consent Form

Patient folder number/hospital sticker

Dear Patient,

You are soon to undergo surgery during which your lymph nodes/glands, that appear to be involved with the melanoma cancer, will be removed. These nodes will, as part of procedure, be sent to the Pathology Department to be analysed. The tissue will then be disposed of appropriately.

We at the Department of Molecular Cell Biology (University of Cape Town), are conducting research that will potentially allow the earlier detection of the spread of the melanoma cancer to these lymph nodes. In other words, in a few years time we might be able to offer patients with early stage melanoma a better chance of survival. However a part of our research requires us to test our protocol on melanoma-involved lymph nodes. We would therefore like to ask you if you would be willing to donate some of your removed glands towards our research. In this way you would be helping in the fight against this melanoma cancer. Before you decide, please feel free to ask your doctor any questions about the research or anything you do not understand in this form.

If you consent/agree to donating this tissue, please fill in the following details:

I (full name) on the(dd/mm/yy)

at (eg. Groote Schuur Hospital) give consent for some of my lymph node tissue removed to be donated to the Department of Molecular Cell Biology (UCT) for the purpose of advancing the research on melanoma.

I understand that the donation of this tissue will in NO way affect the outcome of my condition, nor will the results of the research have any bearing on my condition. However the results of this research will potentially improve the management of future patients suffering with melanoma.

I also understand that my tissue will be used as an example of involved lymph nodes in this research.

Please sign

Thanking you and wishing you all the best,
Dr Virginia Davids (Department Molecular Cell Biology (UCT): 406 6462)

Doctor's full name and signature Date

Witness 1 (name and signature)

(designation)

Witness 2 (name and signature)

(designation).....

To the doctor: Please fill this form out in duplicate for each patient. Place one of these in the patient's surgical folder, and the other please return to:

DR VIRGINIA DAVIDS,
DEPARTMENT OF MOLECULAR CELL BIOLOGY
4th floor anatomy building,
UCT Medical School ph: 406 6462

Thank you for your help towards this research!

A.22 Current patient staging criteria and classification

Table 1. Melanoma TNM Classification

T classification	Thickness	Ulceration Status
T1	≤ 1.0 mm	a: without ulceration and level II/III b: with ulceration or level IV/V
T2	1.01-2.0 mm	a: without ulceration b: with ulceration
T3	2.01-4.0 mm	a: without ulceration b: with ulceration
T4	> 4.0 mm	a: without ulceration b: with ulceration
N classification	No. of Metastatic Nodes	Nodal Metastatic Mass
N1	1 node	a: micrometastasis* b: macrometastasis†
N2	2-3 nodes	a: micrometastasis* b: macrometastasis† c: in transit met(s)/satellite(s) without metastatic nodes
N3	4 or more metastatic nodes, or matted nodes, or in transit met(s)/satellite(s) with metastatic node(s)	
M classification	Site	Serum Lactate Dehydrogenase
M1a	Distant skin, subcutaneous, or nodal mets	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases Any distant metastasis	Normal Elevated

*Micrometastases are diagnosed after sentinel or elective lymphadenectomy.

†Macrometastases are defined as clinically detectable nodal metastases confirmed by therapeutic lymphadenectomy or when nodal metastasis exhibits gross extracapsular extension.

Table 2. Proposed Stage Groupings for Cutaneous Melanoma

	Clinical Staging*			Pathologic Staging†		
	T	N	M	T	N	M
0	Tis	NO	MO	Tis	NO	MO
IA	T1a	NO	MO	T1a	NO	MO
IB	T1b	NO	MO	T1b	NO	MO
	T2a	NO	MO	T2a	NO	MO
IIA	T2b	NO	MO	T2b	NO	MO
	T3a	NO	MO	T3a	NO	MO
IIB	T3b	NO	MO	T3b	NO	MO
	T4a	NO	MO	T4a	NO	MO
IC	T4b	NO	MO	T4b	NO	MO
III†	Any T	N1	MO			
		N2				
		N3				
IIIA				T1-4a	N1a	MO
				T1-4a	N2a	MO
IIIB				T1-4b	N1a	MO
				T1-4b	N2a	MO
				T1-4a	N1b	MO
				T1-4a	N2b	MO
				T1-4a/b	N2c	MO
				T1-4b	N1b	MO
IIC				T1-4b	N2c	MO
				Any T	N3	MO
IV	Any T	Any N	Any M1	Any T	Any N	Any M1

*Clinical staging includes microstaging of the primary melanoma and clinical/radiologic evaluation for metastases. By convention, it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant metastases.

†Pathologic staging includes microstaging of the primary melanoma and pathologic information about the regional lymph nodes after partial or complete lymphadenectomy. Pathologic stage 0 or stage IA patients are the exception; they do not require pathologic evaluation of their lymph nodes.

‡There are no stage III subgroups for clinical staging.

Table 3. Survival Rates for Melanoma TNM and Staging Categories

Pathologic Stage	TNM	Thickness (mm)	Ulceration	No. + Nodes	Nodal Size	Distant Metastasis	No. of Patients	Survival ± SE			
								1-Year	2-Year	5-Year	10-Year
IA	T1a	1	No	0	-	-	4,510	99.7 ± 0.1	99.0 ± 0.2	95.3 ± 0.4	87.9 ± 1.0
IB	T1b	1	Yes or level IV, V	0	-	-	1,380	99.8 ± 0.1	98.7 ± 0.3	90.9 ± 1.0	83.1 ± 1.5
	T2a	1.01-2.0	No	0	-	-	3,285	99.5 ± 0.1	97.3 ± 0.3	89.0 ± 0.7	79.2 ± 1.1
IIA	T2b	1.01-2.0	Yes	0	-	-	958	98.2 ± 0.5	92.9 ± 0.9	77.4 ± 1.7	64.4 ± 2.2
	T3a	2.01-4.0	No	0	-	-	1,717	98.7 ± 0.3	94.3 ± 0.6	78.7 ± 1.2	63.8 ± 1.7
IIB	T3b	2.01-4.0	Yes	0	-	-	1,523	95.1 ± 0.6	84.8 ± 1.0	63.0 ± 1.5	50.8 ± 1.7
	T4a	> 4.0	No	0	-	-	563	94.8 ± 1.0	88.6 ± 1.5	67.4 ± 2.4	53.9 ± 3.3
IIC	T4b	> 4.0	Yes	0	-	-	978	89.9 ± 1.0	70.7 ± 1.6	45.1 ± 1.9	32.3 ± 2.1
IIIA	N1a	Any	No	1	Micro	-	252	95.9 ± 1.3	88.0 ± 2.3	69.5 ± 3.7	63.0 ± 4.4
	N2a	Any	No	2-3	Micro	-	130	93.0 ± 2.4	82.7 ± 3.8	63.3 ± 5.6	56.9 ± 6.8
IIIB	N1a	Any	Yes	1	Micro	-	217	93.3 ± 1.8	75.0 ± 3.2	52.8 ± 4.1	37.8 ± 4.8
	N2a	Any	Yes	2-3	Micro	-	111	92.0 ± 2.7	81.0 ± 4.1	49.6 ± 5.7	35.9 ± 7.2
	N1b	Any	No	1	Macro	-	122	88.5 ± 2.9	78.5 ± 3.7	59.0 ± 4.8	47.7 ± 5.8
	N2b	Any	No	2-3	Macro	-	93	76.8 ± 4.4	65.6 ± 5.0	46.3 ± 5.5	39.2 ± 5.8
IIIC	N1b	Any	Yes	1	Macro	-	98	77.9 ± 4.3	54.2 ± 5.2	29.0 ± 5.1	24.4 ± 5.3
	N2b	Any	Yes	2-3	Macro	-	109	74.3 ± 4.3	44.1 ± 4.9	24.0 ± 4.4	15.0 ± 3.9
	N3	Any	Any	4	Micro/macro	-	396	71.0 ± 2.4	49.8 ± 2.7	26.7 ± 2.5	18.4 ± 2.5
IV	M1a	Any	Any	Any	Any	Skin, SQ	179	59.3 ± 3.7	36.7 ± 3.6	18.8 ± 3.0	15.7 ± 2.9
	M1b	Any	Any	Any	Any	Lung	186	57.0 ± 3.7	23.1 ± 3.2	6.7 ± 2.0	2.5 ± 1.5
	M1c	Any	Any	Any	Any	Other Visceral	793	40.6 ± 1.8	23.6 ± 1.5	9.5 ± 1.1	6.0 ± 0.9
Total							17,600				

Tables 1-3, reproduced from Balch et al (2001)¹⁰

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University of Cape Town

By placing a tick in one box in each group below, please indicate which statements best describe your own state of health TODAY.

Mobility

- I have no problems in walking about 1
- I have some problems in walking about 2
- I am confined to bed 3

Self-Care

- I have no problems with self-care 1
- I have some problems washing or dressing myself 2
- I am unable to wash or dress myself 3

Usual Activities (e.g. work, study, housework, family or leisure activities)

- I have no problems with performing my usual activities 1
- I have some problems with performing my usual activities 2
- I am unable to perform my usual activities 3

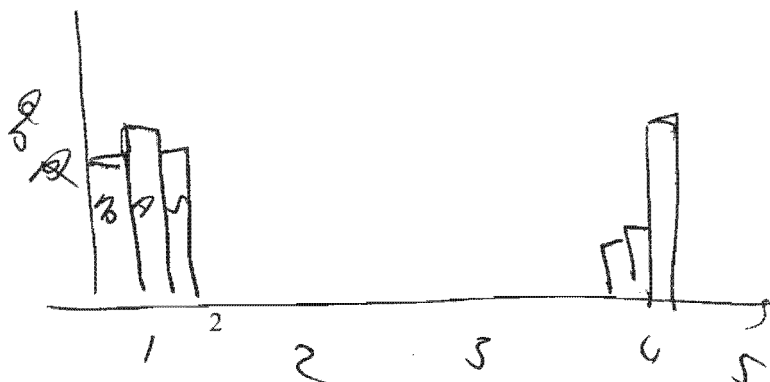
Pain/Discomfort

- I have no pain or discomfort 1
- I have moderate pain or discomfort 2
- I have extreme pain or discomfort 3

Anxiety/Depression

- I am not anxious or depressed 1
- I am moderately anxious or depressed 2
- I am extremely anxious or depressed 3

1 1 1 1 1
1 vs 2 3



To help people say how good or bad their state of health is, we have drawn a scale (rather like a thermometer) on which the best state you can imagine is marked 100 and the worst state you can imagine is marked 0.

We would like you to indicate on this scale, in your opinion, how good or bad your own health is today. Please do this by drawing a line from the box below to whichever point on the scale indicates how good or bad your state of health is today.

**Your own
state of health
today**

