The impact of Synaptophysin-Ki67 and Chromogranin A-Ki67 dual IHC staining in the assessment of the Ki67 Proliferative Index when grading well-differentiated gastrointestinal neuroendocrine tumours (WDGI NETs).

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ABSTRACT

A significant step in the assessment of the Ki67 proliferative index (KPI) when grading well-differentiated gastrointestinal neuroendocrine tumours (WDGI NETs) is the manual counting of active neoplastic cells against the surrounding population of cells. This step is often challenging due to the presence of background stromal lymphocytes and entrapped non-neoplastic glands which can contain proliferating cells. As a result, pathologists can have difficulty distinguishing between tumour cells and non-tumour cells which leads to inaccurate assessment of KPI. This is especially a problem when grading tumours close to the established category cutoffs. Studies show that this problem is challenging to overcome with or without the use of computational tools and automated image analysis to improve assessments made using manual image analysis. Benefits of image analysis solutions are not able to be replicated consistently, leading to a lack of uniformity in reporting. Some solutions were also expensive or not user friendly as they required technical expertise, time and skills to undertake. The aim of this study was to overcome limitations in the Ki67 single stain approach by qualitatively and quantitatively examining the positive impact of dual (or double) immunohistochemistry staining in the assessment of the Ki67 proliferative index (KPI) when grading well-differentiated gastrointestinal neuroendocrine tumours (WDGI NETs).

The study successfully optimized and validated Synaptophysin-Ki67 and Chromogranin A-Ki67 double stain approaches, resulting in a reproducible and reliable methodology for use in a clinical diagnostic setting. The first finding of this study was confirmation that Synaptophysin-Ki67 and Chromogranin A-Ki67 reproduced the results (diagnostic grades) of the gold standard 'Ki67-only' stain. Validation of new procedures is essential for diagnostic accreditation. From a qualitative perspective, the double staining methods were superior in showing the identity of Ki67 positive tumour cells undergoing proliferation. Furthermore, the double stains were significantly more efficient from the perspective of pathologist time. In this regard they outperformed the gold standard method. This was a second key finding to justify further exploration of which double stain method might be the best to adopt. From a quantitative perspective, the double staining methods, on average, produced a less variable assessment of KPI than Ki67 alone. When the subset of tumour biopsy samples was compared with the resected tumour samples, regardless of tumour location in the gastrointestinal system, the KPI assessment results were significantly higher for the biopsy sample subset stained using a dual stain approach. The Ki67 stain alone could not replicate this finding. When the subset of upper gastro-intestinal (GI) tract samples was compared with lower GI tract samples,

regardless of specimen type, the KPI assessment results were significantly higher for the upper GI tract subset using the dual stain approach. The Ki67-only stain could duplicate this trend but without statistical significance. The subset analysis provided further evidence for the utility of the dual stain approach in terms of precision. More research is now required to understand the implications of these differences and if better statistical models are required to understand the performance of biopsies in determining the most accurate diagnosis for WDGI NETs.

The findings of this research will further encourage the use of dual markers to improve the visualization of tumour cells by making it easier to count Ki67 positive nuclei in neoplastic cells, thereby positively benefiting grading assessment for WDGI NETs and improving diagnostic accuracy. This study shows the utility of double stains as a platform for further research. For example, by looking retrospectively at a subset of cases where grade determination and clinical outcomes were in conflict for the Ki67-only stain method and by looking prospectively at how dual stain methods might provide better prognostic and therapeutic recommendations. The goal for future research is to improve case management and patient survival with the input of the oncology and pathology team. Dual stain techniques are very likely to have broader application to neuroendocrine tumours in general.

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List of Abbreviations

Ab	Antibody
AR	Antigen Retrieval
CgA	Chromogranin A
CgA-Ki67	Chromogranin A-Ki67
CT scan	Computed topography scan
DAB	3-3' Diaminobenzidine
DS	Double Stain or Double Staining
DI water	Distilled water
EDTA buffer	Ethylenediaminetetraacetic acid buffer
ER1 and ER2	Epitope retrieval solution 1 and 2
EUS	Endoscopic ultrasound
FFPE tissues	Formalin fixed paraffin embedded tissues
Fc receptor	Fragment crystallizable region
GI NETs	Gastrointestinal neuroendocrine tumours
GI tract	Gastrointestinal tract
G1 G2 and G3	Grade 1, Grade 2 and Grade 3
GI NENs	Gastrointestinal neuroendocrine neoplasms
HRP	Horseradish peroxidase
HPF	High power fields
HIER	Heat-induced epitope retrieval
IgG	Immunoglobulin G
IHC	Immunohistochemistry
KPI	Ki67 proliferative index
MEN1	Multiple endocrine neoplasia type 1
MEN2	Multiple endocrine neoplasia type 2
MRI	Magnetic resonance imaging
NENs	Neuroendocrine neoplasms
NETs	Neuroendocrine tumours
NE system	Neuroendocrine system
NSE	Neuron Specific Anolase
NECs	Neuroendocrine carcinomas
10% NBF	10% neutral buffered formalin
PIER	Protease induced epitope retrieval
PET scan	Positron emission testing
PRRT	Peptide receptor radionuclide therapy
QC	Quality control
r AP	Red alkaline phosphatase
RTU	Ready to use
Syn-Ki67	Synaptophysin-Ki67
SSTR	Somatostatin receptor
SyN	Synaptophysin
WDGI NETs	Well-differentiated gastrointestinal neuroendocrine tumours
WHO	World Health Organisations

1.0 Introduction

Gastrointestinal neuroendocrine tumours (GI NETs) are the most common form of neuroendocrine tumours (NETs) making up approximately 60 percent of all diagnosed NETs cases (1). This subset of NETs arises from the neuroendocrine cells of the GI tract which are mainly distributed in the mucosa and submucosa of the gastrointestinal tract (2). Neuroendocrine tumours are rare types of neoplasms affecting only 6 in 100,000 people worldwide (3), however recent studies show steady increases in the number of new cases being diagnosed every year within the last ten years (4, 5). This trend is most likely due to the arrival of improved diagnostic tests and equipment which can detect NETs early (6, 7). This increase in the number of new cases in Australasia coinciding with the ongoing public healthcare burden of gastrointestinal cancers in the region raised awareness in regional healthcare (8). The risk factors associated with gastrointestinal neuroendocrine tumours are still not well understood making this cancer a healthcare concern worldwide, and signalling more research in the field is much needed. One reason that further investigation of NETs is considered worthwhile arises from the difficulty of assigning a Ki67 proliferation index to the tumour cell population. These tumours often have defensive and stromal host cells surrounding and infiltrating them. These populations may also be proliferating as part of the host reaction but are not from the tumour lineage. This makes it difficult for pathologists to distinguish dividing tumour cells from the surrounding population of active host cells.

Our particular interest is in the grading of well-differentiated gastrointestinal neuroendocrine tumours (WDGI NETs), and specifically in the assessment of the Ki67 proliferative index (KPI). An issue raised by one of our inhouse pathologists, was that it is difficult to distinguish between neoplastic and non-neoplastic cells especially when populations of white blood cells are overrepresented amongst other structural elements of the tumour tissue. This aligns with similar challenges that are well documented in the literature (9-11). Several studies show inconsistencies in the KPI reproduced by different observers when basing assessment of Ki67 proliferative index on the gold standard ‘Ki67-only’ stain. These studies also show that the difficulties to distinguish dividing neoplastic cells are due to the presence of surrounding white blood cells and entrapped non-neoplastic glands which contains proliferating cells (9, 10, 12). This leads to our conceptualisation and proposal to trial the use of double staining methods, combining Synaptophysin and Chromogranin A with Ki67 (a proliferative marker) to highlight tumour cells and the cell division cycle simultaneously. This

approach is hypothesized to make it easier for the pathologists to distinguish proliferation of tumour cells from proliferation of non-tumour cells.

The initial approach and scope for this study was to make comparisons of all staining methods (Ki67, Syn-Ki67 and CgA-Ki67) and KPI determination outcomes available, focusing on the variations in the outcomes of the independent readers. These evaluations of KPI could have been anonymized and limited to the contemporary experiment 'trial runs' or could have included anonymized comparison with previous tumour grade and KPI determinations made at the time the tumour sample was first submitted. However, this was not supported by the leading pathologists for staff resourcing and logistic reasons. Furthermore, a retrospective study may have contradicted the original reported grade (and potentially the diagnosis) if the outcome from the dual stain tests varied significantly from the outcome provided to the patient. This would potentially raise ethical concerns for all groups involved and therefore the trial focussed on technical improvements.

1.1 Aim

This research examined the impact dual immunohistochemical (IHC) stains (combination of Synaptophysin-Ki67 and of Chromogranin A-Ki67) can have in improving the overall grading of well-differentiated gastrointestinal neuroendocrine tumours (WDGI NETs).

1.2 Objectives

1. To evaluate published methods for increasing ease and accuracy of GI NET Ki67 proliferative index.
2. To optimise and validate the gold standard Ki67-only staining method and the proposed double immunohistochemistry (IHC) staining methods; Synaptophysin-Ki67 and Chromogranin A-Ki67 double stains.
3. To assess the reproducibility of the proposed double staining methods using confirmed positive well-differentiated gastrointestinal neuroendocrine tumour (WDGI NETs) cases.
4. To evaluate the influence of different specimen types (biopsy vs resection) from different levels of the gastrointestinal tract (e.g. oesophagus -> stomach →rectum) on the assessment of the Ki67 proliferative index assessment for each of the staining methods.

5. To determine which of the staining methods is more efficient by comparing the time taken by each of the three staining methods to complete a Ki67 proliferative index assessment.

We hypothesized that the double stains would not only highlight the tumour cells, making them more visible and easier to identify, but also prevent the Ki67 proliferative index being over estimated due to inclusion of dividing cells that were not neoplastic.

We expected that not only would these proposed staining methods replicate similar results to those generated by the gold standard but could also do better. We also hypothesized that conceptually, dual staining would improve the assessment of the KPI by reducing the assessment time (amount of time spending per case) and pathologist resources without affecting accuracy and precision in grading well-differentiated gastrointestinal neuroendocrine tumours.

This study focussed on well-differentiated NETs because of their slower growth and spread. Accurately grading the main tumour before it has reached and acquired advanced N (lymph node) or M (metastasis) scores is associated with better patient outcomes (23). We reasoned that accurate grading of well-differentiated tumours would provide patients with a better chance of achieving complete remission. Assessment towards a potential surgical ‘cure’ would bring the best outcomes.

2.0 Literature Review

2.1 Neuroendocrine Tumours (NETs)

Neuroendocrine tumours (NETs) or neuroendocrine neoplasms (NENs) are a rare heterogeneous group of tumours with disparate histology and classification system that arises from the cells of the diffuse neuroendocrine system (5, 13). The neuroendocrine (NE) system is comprised of endocrine glands (pituitary and parathyroid glands etc), endocrine islet tissue embedded within glandular tissue (thyroid or pancreatic), as well as cells within exocrine parenchyma (endocrine cells of the digestive and respiratory tracts) (14). According to the 2022 World Health Organisation (WHO) Classification of Endocrine and Neuroendocrine Tumours, NETs are categorised into well-differentiated neuroendocrine tumours and poorly differentiated neuroendocrine carcinomas (NECs). This classification is based on tumour cells differentiation thus NETs are generally (but not always) graded as G1, G2 and G3 and NECs are high grade or poorly differentiated (13). This type of neoplasm was previously classified in the old classification system into two types: carcinoids and endocrine pancreatic tumours depending on the anatomical location of the primary tumour. Carcinoids were further subdivided into foregut, midgut and hindgut depending on their embryonic origin. The foregut carcinoids are made up of the neuroendocrine tumours from the lungs, thymus, stomach, and duodenum while the appendix, jejunum, ileum and proximal colon tissues encompass carcinoids from the midgut. The hindgut carcinoids originate in the distal colon and rectum (6). Neuroendocrine tumours can originate from any part of the body, but the most common sites are the gastrointestinal tract (62%-67%), lungs (22%-27%) and the pancreas (approx.12%) (1, 15). Neuroendocrine tumours are rare because they account for only 2 percent of all newly diagnosed malignancies (3). Studies shows that the incidence of NETs in Australasia and the rest of the world have been steadily increasing in the last 3 to 4 decades (16) with the greatest rates of increase in the USA, Canada and Norway (1, 3, 17). In the United States alone, the prevalence of the disease between 2012 to 2017 was approximately 170,000 patients and the number is expected to be a lot more in the future (18). Gastrointestinal neuroendocrine tumours (GI NETs) make up a small fraction of all colorectal cancer cases and are a burden to healthcare in New Zealand (8) and around the world. These types of tumours are slow growing with a 5-year overall survival rate of 70-80 percent. The chance of surviving the disease is strongly dependent on the tumour stage at diagnosis with studies revealing 20-45 percent of NETs having metastasized at diagnosis (8, 16). Early diagnosis can improve the 5-year overall

survival rate to 90 percent. Unfortunately, early diagnosis is not always the case as these tumours have mostly gone undetected even when accompanied by common symptoms such as stomach pain, diarrhoea, hot flushes, bloating, prolonged wheezing or coughing, heartburn and weight changes, all of which can indicate other health conditions (1, 16). In the 2017 international survey of 1928 patients by Singh and colleague, the authors found a mean delay of 52 months between symptom onset and diagnosis with; patients usually going through more than five different clinicians before receiving the correct diagnosis (19). Depending on the type of NETs suspected, clinicians have a broad spectrum of diagnostic methods to choose from which includes; biopsy, core needle biopsy, urinalysis, biochemical tests, computed tomography (CT) scan which is used to create 3D images of the tumour, magnetic resonance imaging (MRI) and positron emission testing (PET) scan (20-22). The treatment options for NETs depends on a variety of factors such as the types/class of NETs, tumour site/location, symptoms due to excess hormones produced by the tumour and side effects of the treatment itself (21, 23). Clinicians can choose from the following; surgery to remove the entire tumour and some of the healthy tissues around it; chemotherapy to destroy tumour cells; targeted therapy; peptide receptor radionuclide therapy (PRRT); medications to control excess hormones; and radiation therapy (20, 24).

2.2 Gastrointestinal NETs (GI NETs)

Gastrointestinal neuroendocrine tumours (GI NETs) are the most common form of NETs making up approximately 60 percent of all diagnosed NETs cases (1, 2). They are also known as gastrointestinal neuroendocrine neoplasms (GI NENs). They arise from the neuroendocrine cells of the GI tract which are mainly distributed in the mucosa and submucosa of the gastrointestinal tract (25). These cells possess both neural and endocrine characteristics (14). In the cytoplasm of neuroendocrine cells are dense core granules which synthesize and secrete chromogranin A (CgA), synaptophysin (SyN) and Neuron-Specific Enolase (NSE), the three glycoproteins most often associated with NETs and essential for the diagnosis of NETs (14). Serum CgA is the most often used tumour marker to assess disease burden and monitor treatment response as CgA is elevated in both functional and non-functional NETs (2). Another essential prognostic indicator for the management of NETs is called Ki67. This biomarker is not specific for NETs but is found in the nucleus of all cells and expressed during the cell division cycle. The abundance of Ki67 is correlated with cellular proliferation, a proxy for how fast tumour cells is dividing, and influences the histological grading of NETs and ultimately prognosis (1, 5, 14).

2.3 Epidemiology of GI NETs

The number of diagnosed gastrointestinal neuroendocrine tumours has been increasing for years (1, 3). This is thought to be related to improvements in the way GI NETs are currently diagnosed with the help of advanced and improved imaging tests and endoscopy. Furthermore, there is increased awareness of these types of tumours (16, 21) and this is reflected by the number of new cases diagnosed in the US alone, increases from 1.09 to 6.98 per 100,000 population per year (4). Similar trends have been observed in the Australasia in the last ten years (8, 16). The most common places in the GI tract for this type of tumour are the small intestine and rectum (4). The 5-year relative survival rate for GI NETs is 94% and it varies between patients depending on the following factors; the stage/grade of tumour, age and general health of patients, location of the tumour in the GI tract, and effectiveness of any previous treatment plan provided (8). The 5-year relative rate for people with a gastrointestinal NET that has not spread to other parts of the body is 97% (4, 8). If the tumour has metastasised and spread to distant areas of the body via the lymphatic system, then the relative 5-year survival rate reduces to 68% (4, 8). Furthermore, GI NETs are a public health challenge due to risk factors that are not being fully understood. Previous studies strongly associate the neoplasm with a family history of cancer (26, 27). Other risk factors such as obesity, excess alcohol consumption and cigarette smoking can also increase the risk of getting neuroendocrine tumours (27).

2.4 Symptoms/Diagnosis of GI NETs

Most GI NETs cases are asymptomatic and therefore diagnosis is mostly incidental (2). A large proportion of the cases are discovered during endoscopy to investigate underlying causes for other health issues or conditions such as unexplained and non-specific abdominal problems and/or pain, anaemia and indigestion (28). Symptomatic GI NETs patients are known to present varieties of symptoms which depend on a number of factors such as hormones, tumour size/growth and metastasis (29). The variations in symptoms between cases of GI NETs makes it difficult to diagnose the tumour early. Symptomatic GI NETS patients often presented with symptoms such as diarrhoea, flushing, steatorrhea, wheezing and peptic ulcers (29). Other symptoms such as abdominal pain, feeling sick in general, breathlessness, looking pale (due to anaemia), unexplained weight loss and melaena or haematemesis can be also experienced by patients due to excess growth of the tumour and pushing against other organs in the body (20, 21, 29). These symptoms can be also seen in other health conditions which can make it difficult

to diagnose and manage GI NETs effectively as a single disease entity in general medicine (21, 29).

The diagnosis of GI NETs requires a coordinated multidisciplinary effort involving medical oncologists, surgeons, interventional radiologists and pathologists. Results from pathology testing, hormonal testing, diagnostic and functional imaging are integrated to form a comprehensive holistic picture (20). Based on the physical and clinical symptoms presented by the patient, family history is then being looked at to decide whether there is a need for physical examination performed or not. Physical examination includes requesting a blood test, urine test, medical imaging and a biopsy (20). There are several medical imaging tests available which include CT scan, MRI scan, endoscopic ultrasound (EUS), and radioactive scans (20). A biopsy is mandatory for the diagnosis of all types of neuroendocrine tumours including gastrointestinal neuroendocrine tumours (20).

2.5 Classification of GI NETs

Regardless of the location of the tumour in the gastrointestinal system, the classification of the GI NETs will always be consistent when it is based on the 2022 World Health Organisation (WHO) Classification system of Neuroendocrine Tumours. This classification system is based on cellular proliferation assessment utilizing the mitotic rate, Ki67 labelling index and tumour cellular differentiation (13). The mitotic rate and Ki67 labelling index makes up the grading system of the disease, stratifies NETs into grade 1 (G1), grade 2 (G2), and grade 3 (G3), corresponding to low-grade, intermediate-grade, and high-grade categories (13, 29). In general terms, low-grade GI NETs are malignant neoplasms in slow motion whereas high-grade ones are aggressive and metastatic. Intermediate-grade GI NETs have a less predictable course (20). The 2022 WHO Classification system also categorises GI NETs into well-differentiated (neuroendocrine tumour, NET) and poorly differentiated (neuroendocrine carcinoma, NEC) based on the morphology of the cells (2, 13, 29). Tumour cell differentiation refers to the extent to which the neuroendocrine tumour cells resemble healthy neuroendocrine cells (6). Neuroendocrine tumour cells possess characteristic pathologic features derived from a neuroendocrine lineage and they resemble their non-neoplastic cell counterparts. These tumour cells typically contain many neuroendocrine secretory granules in the cytoplasm, which is reflected in the diffuse and strong immunoreactivity for neuroendocrine differentiation markers, along with frequent hormone and somatostatin receptor (SSTR) expression (2, 30). Poorly differentiated NECs seldomly resemble any non-neoplastic cell counterparts and

cytomorphological features are usually atypical, consistent with high grade categorization, and with reduced or absent hormone and SSTR expression (13). Neuroendocrine Carcinomas also exhibit small cell and large cell phenotypes with characteristic morphological features. The grading of the tumour (based on mitotic rate and Ki67 proliferative index) in contrast to cellular differentiation explains the phenotype of the tumour with its ability to grow fast and spread from its site of origins (13). The technicalities of how grading is assessed and measured histologically will be discussed in detail in the next section.

2.6 Histological Grading of GI NETs by assessing Mitotic Rate

Accuracy in the grading of NETs is fundamentally important as it is one of the key determinants of prognosis. The grade of the tumour reflects the biological aggressiveness of the neoplasm and provides key information to the clinician regarding GI NETs patient care and management (20). As stated above, the WHO classifies GI NETs into two main categories: well-differentiated GI NETs and poorly differentiated GI NETs. Well-differentiated GI NETs are further categorised into three subgroups based on their histological grades and they are: G1, G2 and G3 (2). The WHO classification system of tumour grading uses mitotic rate and/or Ki67 index to determine the grade of a tumour and its potentially aggressive behaviour. The mitotic rate or mitotic count is usually reported as the number of mitoses per 2 mm^2, done by evaluating at least 10 mm^2 in the most mitotically active part of the tumour (called the HOTSPOT). Only clearly identifiable mitotic figures should be counted; hyperchromatic, karyorrhectic, or apoptotic nuclei are excluded. Because of variations in field size at 40x magnification, the number of high-power fields (HPF) required to evaluate 10mm^2 must be determined for each microscope (Appendix 1). For instance, if using a microscope with a field diameter of 0.55 mm, the number of mitotic figures in 42 HPFs is counted, and the resulting number is divided by 5 to determine the number of mitoses per 2 mm^2. This is the mitotic count and is assigned to determine tumour grade. A mitotic count of less than 2 is considered Grade 1; 2 to 20 is Graded 2 and any tumours with more than 20 dividing cells/ 2 mm^2 considered as Grade 3 (Table 2.1 below) (2).

2.7 Grading using Ki67 Proliferative Index (KPI) Assessment

The grading of gastrointestinal neuroendocrine tumours can be also accomplished through assessment of the Ki67 Proliferative Index (KPI). KPI is reported as **percentage positive tumour cells (% positive tumour cells)** in the area with the highest nuclear labelling (HOTSPOT), although the precise method of assessment has not been standardized. The most

crucial challenge in the assessment of KPI is the counting of the positive tumour cells against surrounding non-neoplastic cells. Several cell counting methods have been used including automatic counting and eyeballing (15). The eyeballing method is a challenge itself as it is an educated guess based on numerous times of performing manual counts in different varieties of tumours. Automated methods for measuring Ki67 proliferative index utilize digital analysis and deep learning to quantify the percentage of cells expressing Ki67 (31). These methods are fairly new and have struggled with accuracy and reproducibility due to several factors including; heterogeneity of the tumours, difficulty distinguishing tumour cells, subjectivity in defining hotspots and staining intensity, lack of standardization and the need for AI refinement(11, 12). The current AI system recently introduced in the field to quantify Ki67 index in NETs (31, 32) is a developing field and may not be able to replicate the nuanced judgement of experienced pathologists. Future research and development are therefore required to refine AI algorithms and make them more robust and reliable for GI NETs. In contrast, eyeballing can be used for most tumours; however, for tumours with a Ki67 proliferative index close to the grade cut-off, it is recommended to perform the counts manually on a printout of camera-captured images of hot spots (12). It has also been recommended that a minimum of 500 tumour cells be counted to determine the Ki67 index, and a note made if fewer cells are available. When both methods are used, the grade of the tumour is usually based on the Ki67 index (11, 12, 15). Many clinicians and studies found the use of KPI more reliable than mitotic counts for several reasons (10, 15, 33, 34). The number of tumour cells evaluated is dependent on the density of tumour cells when the hotspot area is defined by mm^2 as the stromal cell and matrix density can vary between and within tumours. Percentage of dividing tumour KPI picks up cells entering the division cycle but that have not yet progressed to showing separated forms of nuclear chromatin (12, 15). As such mitotic figures underrepresent the true rate of proliferation, and this makes mitotic rate less reliable.

GI NETs tumours with a Ki67 index of <3% are classified as Grade 1, those with 3-20% are classified as Grade 2, tumours with Ki67 index of greater than 20% are Grade 3. Grade 1 to Grade 3 NETs (low grade) are well differentiated tumours, and the cells look more like normal cells (Table 2.1 below) (13).

Table 2. 1 WHO Grading System for Well-differentiated gastrointestinal neuroendocrine tumours.

Grade	Mitotic Rate (per 2mm²)	Ki67 Index (%)
Well-differentiated neuroendocrine tumour G1	<2	<3
Well-differentiated neuroendocrine tumour G2	2-20	3-20
Well-differentiated neuroendocrine tumour G3	>20	>20

2.8 Prognosis and Patient Management

Traditionally, following a diagnosis for NETs (GI NETs in this case) the care team would work with the patient to develop a comprehensive holistic treatment plan based on the diagnosis and patient needs. As stated above, most NETs cases present at an advanced stage, so an integrative approach will be key for longer-term patient survival. Key information required in this process includes the grade and stage of the disease which will help in planning the most appropriate treatment for the patient. In the case of poorly differentiated gastrointestinal neuroendocrine carcinoid tumours, the stage of the disease is important as it shows how far the cancer has spread in the body. Staging of gastrointestinal neuroendocrine carcinoid tumours follows the American Joint Committee on Cancer (AJCC) TNM system which is based on the size and extent of the main tumour (T), spread to nearby lymph nodes (N), and tumour metastasis (M) to distant parts of the body. These three metrics generate a score ranging from 1 to 4 which indicates how advanced the disease is with the highest number of 4 showing a more advanced cancer. (23). In contrast, well-differentiated gastrointestinal neuroendocrine tumours have a better prognosis, as they generally grow slower than poorly differentiated carcinoid tumours and often offer a wider range of treatment options and a better chance of successful outcomes.

There are several treatment and therapy options available for patients with gastrointestinal neuroendocrine tumours. The following treatment options are available for NETs patients (including GI NETs patients) in NZ; surgery (8), chemotherapy, targeted drug therapy, peptide receptor radionuclide therapy (PRRT), medications to control excess hormones, and radiation therapy (8, 20). While the stage of the tumour is a critical component of survival time, the grade of the tumour is also strongly factored and essential in clinical decision-making for patient care,

especially in relation to treatment options. This is alongside other factors which are also important to be considered when determining the best effective treatment options for each GI NET patient. These factors include the location of the tumour for surgical accessibility, the physical health of the patient, the availability of surgery and patient preferences. The treatment plan varies from patient to patient, and it is a collective effort of a multidisciplinary care team instead of a single clinician. The goal is to put together a treatment plan that balances the goal of eradicating the cancer while preserving patient quality of life (35).

2.9 Challenges in the Assessment of Ki67 Proliferative Index

Based on the observations of two of our inhouse pathologists, a common challenge in the assessment of Ki67 proliferative index in well-differentiated gastrointestinal NETs is the difficulty of distinguishing between "tumour cells and everything else". Accuracy in lab test results is fundamental to patient care and a false positive lab test result will be a disadvantage. In the worst-case scenario, the patient may receive unnecessary treatments such as radiotherapy or chemotherapy because of a false positive test result.

The assessment of Ki67 Proliferative Index involves counting the number of positive tumour cells in areas with the highest nuclear labelling also known as the hotspot. The counting of the tumour cells is usually done with a method called "eyeballing"- which is the manual counting of the tumour cells on a camera-captured image of the hotspot. The counting of these tumour cells can be quite challenging at times due to the presence of background stromal lymphocytes and entrapped non-neoplastic glands which often contain proliferating cells. This subset of proliferating cells also stained positively for Ki67. Neuroendocrine tumour characteristically has a delicate vascular network with capillaries growing into and surrounding the neoplastic tissue. This provides another subset of proliferating cells which can also resulted in false positive staining for Ki67. These non-neoplastic cells can be difficult to morphologically distinguish from tumour cells with the Ki67 stain and, if mistakenly counted, can artificially elevate the proliferative index. Furthermore, in small biopsies, a cautery artifact can alter the morphologic appearance of tumour cells, making the Ki67 proliferative index more challenging to assess. These challenges often resulted in either overcounts or undercounts of the neuroendocrine tumour cells and/or lack of consensus which can easily alter the grading of the tumour. The threshold between grade 1 and grade 2 GI NETs is quite low and undercounts or overcounts by one or two tumour cells could result in the wrong grade being assigned. This outcome will likely affect patient management in a negative way. The observations of our

pathologists align with difficulties raised in recent similar studies on gastrointestinal neuroendocrine tumours which strongly encouraged this study to take place (9, 10, 33)

2.10 Tumour and Proliferation Markers (Ki67, Syn and CgA)

The two tumour markers of interest in this research are Synaptophysin (SyN) and Chromogranin A (CgA). Ki67 is a known proliferative marker. It is an established marker of cell proliferation and an important prognostic factor for Neuroendocrine Tumours (NETs) including GI NETs. Ki67 antibodies mark nuclear antigens that present in all stages of the cell cycle, except in the resting phase G0 (7, 36) which increases its value as a prognostic tool. Traditionally, the grading of gastrointestinal and pancreatic NETs was based on mitotic index or mitotic rate, which is the number of mitotic figures per 10 high-power fields at 400 x magnification (11). However, this method only identifies proliferating cells in the mitotic phase (7). The mitotic index is with distinct chromatin patterns less able to represent the extent of cell proliferation. Synaptophysin (SYN) and Chromogranin A (CgA) are the two most widely used IHC markers for neuroendocrine cells and their corresponding tumours, including GI NETs (37). They are also currently considered as the most specific immunohistochemical markers for all neuroendocrine neoplasms (9, 38, 39). These two glycoproteins are widely expressed and found in the cytoplasm of normal neuroendocrine cells of the GI tract as well as in many of their corresponding tumours (40). These cytoplasmic IHC markers are used to confirm the origin of GI NETs.

2.11 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is an invaluable laboratory technique that allows cellular proteins and related antigenic macromolecules in tissue samples to be labelled or marked utilizing the interaction between a primary antibody/target antigen and detection reagents. For decades, immunohistochemistry stains have been routinely used on the bench, and many scientists are proficient with the manual staining methods. The shift to auto staining instruments like the use of the current Leica Bond 3 and Bond Max Platforms (machines) allows increased workload throughput while still maintaining consistency, reproducibility and quality of staining. Despite this, each protocol used on any of the auto stainers must be optimized and validated to obtain accurate, consistent and reproducible test results. This is especially important when performing a double staining such as the two proposed double staining trial in this study.

Immunohistochemistry utilizes a specific binding approach to target tissue markers of interest, based on antigen-antibody interactions coupled to a detection system which then visualizes where this interaction has taken place (41, 42). The location of the interaction is usually visualized by secondary means, such as a fluorescent output or a chromogen produced by a coupled enzyme (43). There are two methods of IHC staining: direct and indirect IHC (41-43). **Indirect IHC is the commonly used method in diagnostic labs. It is also the method of choice for this research**. The technique involves the use of an unlabelled primary antibody (first antibody) against target antigens in the tissue and a labelled secondary antibody. The labelled secondary antibody binds with the primary antibody and incorporates the means by which- the primary antibody and antigen of interest is visualized under the microscope (Figure 2.1, below) (41).

Figure 2. 1. Direct and Indirect Immunohistochemistry Techniques.

In both techniques of immunohistochemistry, the targeted antigens in the tissues can be visualized by the pre-labelled primary antibody binding directly to a target via a pre-labelled secondary antibody that recognizes the primary antibody. This latter situation is termed indirect because the primary antibody is unlabelled. The unlabelled primary antibody can only be detected by the addition of a second-tier reagent. In this diagram the comparison is made using a fluorescent label. More commonly in diagnostic IHC the label is an enzyme, and this enzyme produces a permanent-coloured product to mark the location of the target protein.

2.12 Double (Dual) IHC staining

The application of multiple chromogenic IHC staining protocols (dual and triple IHC stains) is on the rise in diagnostic settings such as the use of the Ki67/p16 for the detection of a risk of cervical cancer in women (44) and prostate cocktails (a triple stain combination of CK5, CK14 and p63) & (CK5/14 + p63 + P504S) used for the detection of prostate carcinoma (45). The technique allows simultaneous detection of multiple different protein markers on a tissue

section or a cell, providing a more comprehensive picture of cellular interactions within a tissue sample by visualizing several antigens at once. Double IHC staining methods follows the same principle as that of the indirect IHC, however utilizes two sets of antibodies and reagents to target two different antigens of interest. One antigen in the nucleus and the other in the cytoplasm (i.e. Ki67 antigens in the nucleus and the Syn in the cytoplasm) (Figure 2.2) without overlapping.

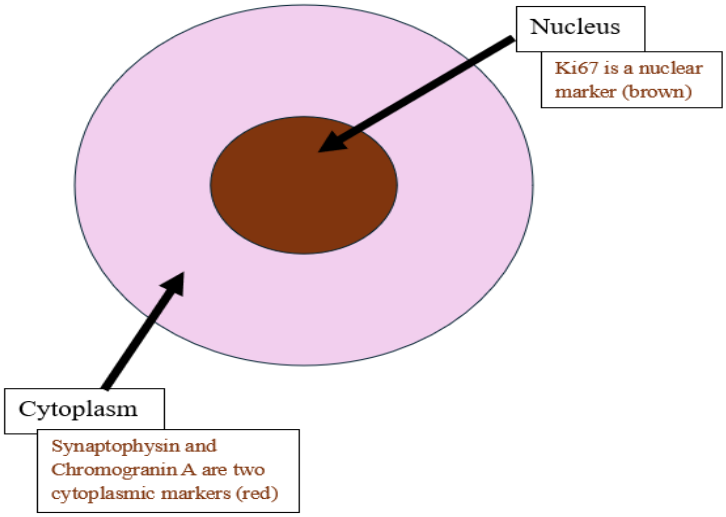

Figure 2. 2. Nuclear marker vs Cytoplasmic marker.

Double IHC staining is known to be either a sequential staining process or simultaneous. The sequential staining process is usually long and requires about 40 to 60 additional minutes per antigen (46). Each antigen is labelled one after another in a sequence (Figure 2.3) making it less efficient to be used in diagnostic labs regardless of the laboratory workload. It is important to mention this and this was the method adopted in the study of Matskuma and colleagues (9) where no cocktails were made. The antibodies (Synaptophysin and Ki67) were applied separately. We opted not to follow this and do the simultaneous staining process instead.

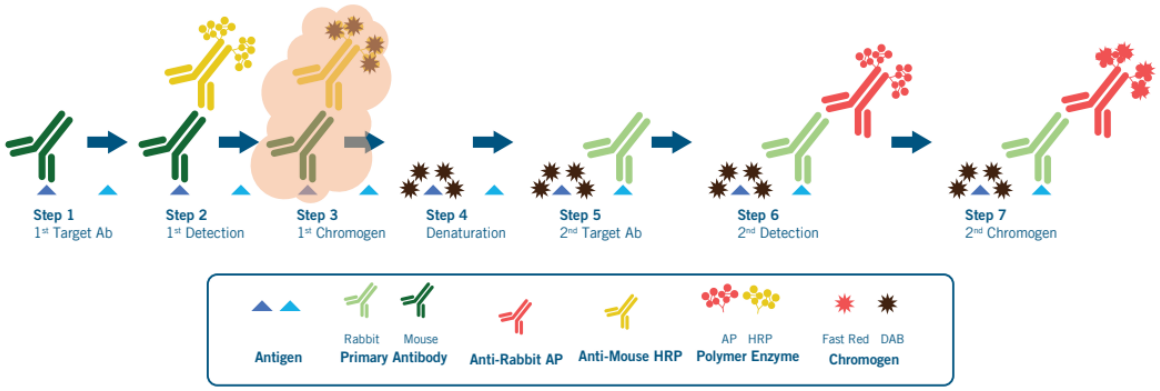

Figure 2. 3. Double sequential IHC staining process.

This type of Double IHC staining is usually long labelling one antigen at a time in a sequence. Not fit for diagnostic laboratories. A denaturation step of the first antigen is also required before the addition of the second primary antibody.

The simultaneous staining process is faster and therefore suits the quick turnaround times in diagnostic laboratories. Simultaneous staining typically uses cocktailed primary antibodies and detection system like our inhouse Chromoplex 1 refine detection system which reduced protocol steps and overall turnaround time (Figure 2.4). A typical staining protocol for simultaneous staining consists of an epitope retrieval, one primary cocktail incubation, one cocktail secondary antibody incubation, 1st chromogen incubation and followed by the 2nd chromogen incubation (Figure 2.4).

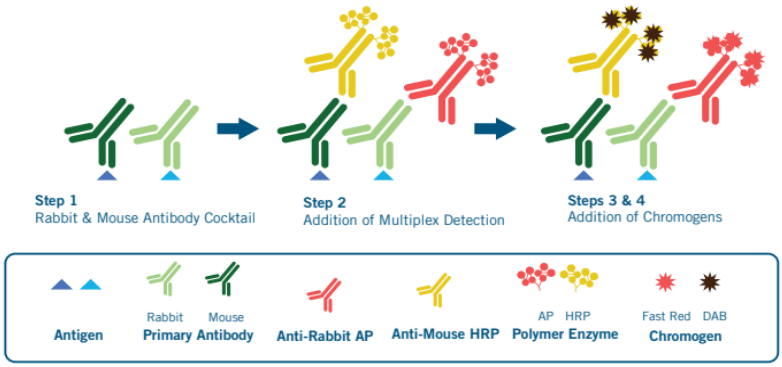

Figure 2. 4. Simultaneous Staining Protocol.

The cocktailed primary antibodies can be made up of more than one antibody. In this study the primary antibodies Synaptophysin and Chromogranin A will be mixed with the nuclear marker Ki67 plus diluents. Other multiple IHC staining antibody cocktails like the triple stain Prostrip (CK14, CK15 and p63) can have up to three antibodies. In theory, the primary antibodies combining in the cocktail must be from two different sources i.e. one should come from a mouse and the other from a rabbit.

2.13 Evaluation of Immunohistochemistry Stain (Scoring System)

A scoring system is required in Immunohistochemistry to convert subjective perception of IHC-marker expression by histopathologists into meaningful quantitative data which is then used for either statical analyses/ diagnostic report and establishing of the conclusions in a research or diagnostic results (47). Without a scoring system, the description of the data being collected can be provided only with subjective perception, expressed in such adjectives as "strong", weak, "absent" with modifiers as "more" or less. The selected scoring (evaluation) system must be definable, reproducible and must create meaningful results. Several scoring systems have been utilized in histopathology but there has not been a widely accepted scoring system for immunohistochemistry yet. Some of the scoring system available includes the following: description of morphological parameters; evaluation of number of IHC-positively stained cells and structures; evaluation of IHC-positively stained cells and/or area ratio; qualitative scoring; and the combinative semi-quantitative scoring approach. Selection of which approach to use depends solely on the research or the pathologist. This study used the qualitative scoring method which is commonly used by scientists (47). Scientists and researchers often use this method to interpret IHC staining in different investigated areas of interest like the hotspots in this study. Score ranks usually lie in a range from negative to positive which may be signed with different amount of "+" depending on how many other categories lay between these border parameters (48-50).

2.14 Optimization and Validation of Antibodies in IHC

Any antibody brought into the laboratory must be optimized and then validated to ensure reproducibility and consistency in results. Primary antibodies should be optimized prior to validation. Optimization of primary antibodies includes finding the appropriate dilution (for concentrated antibodies), optimal antigen retrieval method, and antibody incubation time (51, 52). It is common for manufacturers to send primary antibodies together with an antibody product insert sheet (Appendix 2-4) that includes recommendations for the starting point of optimising dilution and appropriate control tissues. This helps histology technical staff reduce their workload by not having to trial too many different protocols before converging on optimal settings. Without using these guidelines, the process can be time consuming and costly in terms of the reagents themselves (9). Control tissues used in optimization should be fixed and processed identically to patient tissue (52). After antibody optimization is complete, validation of selected staining parameters is achieved by performing IHC staining with the new antibody

on a set number of slides that include sections with a wide range of reactivity (strongly positive, moderate to weak and very weak). The main parameters of the optimisation and validation process are further discussed in detail in the next few sections.

2.14.1 Choice of primary antibodies

The selection of primary antibodies depends on several factors. Firstly, the antibodies of the antibody cocktail must be from different sources i.e. Synaptophysin and Chromogranin A are the cytoplasmic markers (rabbit host) and the nuclear Ki67 marker (mouse host). Secondly, it is important that these markers had been used in study documented in the literature and proven to work on FFPE tissues. Thirdly, they must be accessible and affordable. Pre-diluted antibodies are ready/easy to use because as they are already being optimized and validated by the manufacturer. However, they do not offer the longest shelf life or lowest cost. Purchase of concentrated antibodies is more cost effective, but it takes more time to perform antibody titrations and optimize over a series of dilutions. (51).

2.14.2 Titration of concentrated antibodies

Different antibodies have varying affinities for their target antigen, and even the same antibody can behave differently depending on the experimental setup. This makes finding the optimal dilution crucial for reliable, accurate, consistent and reproducible outcome. This process always started off with a recommended dilution suggested by the manufacturer as starting points. The technician or scientist can then create a series of dilutions around that point, typically 2 or 3-fold increments. There are factors to consider when optimizing antibody dilutions for instance; monoclonal antibodies require higher dilutions compared to polyclonal antibodies; the concentration of the target antigen in the sample will influence the optimal antibody dilution; the detection methods may require different antibody dilutions; and lastly the blocking buffer used can impact background staining, so optimizing this alongside antibody dilution is important.

2.14.3 Antigen retrieval

Antigen retrieval is usually the first step in immunohistochemistry which involves unmasking antigens cross-linked during formalin fixation of tissues. The technique has significantly increased the sensitivity of immunohistochemistry and consequently greatly expanded its application over the years (53). Although formalin fixation of tissues is essential for the preservation of tissue and cellular morphology, the process can also negatively impact immunohistochemistry detection of antigens (42). It does this by cross-linking amino acids within the antigens (epitope) and/ or altering the electrostatic charge of the antigen. The need for antigen retrieval depends on multiple variables, including but not limited to the target antigen, the antibody used, the type of tissue, and the method and duration of fixation (42). There are various methods of antigen retrieval depending on the specific target antigen, and antibody but most involve the breaking of the linkages formed between chemically modified amino acid sidechains and other reactive functional groups within tissues. Linkages that are formed by fixation can be reversed with chemical or physical means (54). Physical treatments include heat and ultrasound while chemical methods include enzyme digestion (54). The methods can be used together in some applications where enzyme and heat treatment are both being utilized. The two most used methods of antigen retrieval are: protease-induced epitope retrieval (PIER) and heat-induced epitope retrieval (HIER). Both methods need to be optimised well to ensure efficiency in immunohistochemistry applications.

The mechanism involved in the heat treatment of tissue in antigen retrieval is not fully understood. However, heat-induced hydrolysis resulted in the relaxation or breaking of formalin-induced cross-linkages (55, 56) . It is believed that calcium ions are also involved in the cross-linking of antigens through the production of hydroxymethyl groups (55). To remove these calcium ions during antigen retrieval, buffered salts solutions based on citrate, phosphate, tris or EDTA are commonly included in the composition of the aqueous antigen retrieval solution needed for heat-based antigen retrieval (55, 57)

2.15 Conclusion

Assessment of the Ki67 proliferative index in WDGI NETs is a challenge due to difficulties differentiating tumour and non-tumour cells. Proposal to boost accuracy using computation tools, automated image analysis and manual image analysis were found to be either inconsistent, lacking uniformity, expensive and not user friendly as they required technical expertise and skills to operate. As a result, we adopted the use of the double immunohistochemistry staining concept to highlight both tumour and proliferating cells making it easier to assess proliferation in the neoplastic population (NP). Ki67 proliferative index is essential for tumour grading of well-differentiated neuroendocrine tumours and therefore mandatory that it is done accurately. The assessment of KPI as discussed above involves the manual count of both tumour cells and non-tumour cells in a hot spot or highest area of nuclear labelling. In certain circumstances, for instance, in small biopsies, crush and cautery artifact these can alter the morphologic appearance of tumour cells and make the manual counting of the cell more challenging. The application of double immunohistochemistry staining is on the rise to make diagnostic processes involving histology more accurate and quicker to perform. Using the dual staining on GI NETs, allows proliferating cells to be more accurately placed into a category of dividing tumour cell or dividing adjacent cell (i.e. a tumour associated cell but not from the cancer cell lineage). This research will optimize the antibody markers and validate the process that allows for this categorization of Ki67 positive cells. Furthermore, it will enable comparison of previous and future approaches to diagnosis for accuracy, efficiency and cost effectiveness.

3.0 Validation and optimisation of the Staining Methods

3.1 Introduction

The validation and optimisation of primary antibodies before they are used as diagnostic tools in laboratories is a significant step in quality control and frequently a challenging, multistep process. There are several factors which can influence the optimization of primary antibody binding as revealed by a fixed chromogen detection system selected. The most influential factors are tissue fixation, targeted antigen of interest (i.e. Synaptophysin, Chromogranin A and Ki67) antibody concentration/ dilution, antigen retrieval methods and control selections. Some variables would be expected to exert a relatively minor influence and are not able to be changed, for example the laboratory hardware platform itself (BOND III) and consumables that are designated by the manufacturer. The Leica detection system kit used in this study has been optimised for use on any of the automated BOND platforms (Appendix 5&6) by its manufacturer Leica. This BOND polymer refine detection kit contains a peroxide block, post primary secondary antibody, a polymer reagent, DAB chromogen and a haematoxylin counterstain. While this kit does not need to be re-optimized internally in the lab as it is a ready to use detection kit some variable elements of manual IHC optimisation are more difficult to vary on automated platforms, for example, adjusting chromogen development time. In the context of this optimisation the instrument is a constant and the detection system is a variable that comes with some instrument and manufacturer specified boundaries.

Selection of appropriate controls for any antibodies being optimized can be aided with the help of the manufacturer insert sheet (also called a datasheet) which came with the antibodies or from reviewing of the literature. The use of a positive control is mandatory in immunohistochemistry staining and very often a negative control is also used alongside the positive control. Negative controls are important during the optimization process to identify and eliminate non-specific staining. Once a protocol is optimized the routine use of negative controls may not be required. Although batch controls, the use of a single positive and negative control, is an option to save on expenses and time, most laboratories in the country still prefer the use of appropriate positive controls to be placed on the same slide as that of the test. This is because all of the automated IHC staining platform including the BOND machine is staining individual slides instead of batch staining. It is important in optimisation that the same experimental conditions are being applied to both the test and control sections.

Tissue fixation is a dynamic and continuous process that results in successful penetration of the tissue by fixatives. It is a crucial process in histopathology preserving cellular structures and composition of tissues so they can be studied or evaluated diagnostically. Tissues used in this study were fixed in 10% formalin resulting in the formation of methylene bridges cross linking the proteins i.e. epitope structures are bridged to adjacent proteins and made cryptic. This masks the antigens from the primary antibodies to a variable extent, and it is extremely common for primary antibodies not to bind to target structures if they are not unmasked or retrieved. This makes antigen retrieval a crucial step where methylene bridges get broken up and some of the additive chemical is removed from the section, restoring more native functional groups, exposing the antigenic sites and thereby allowing the antibodies to bind. HIER was the method of choice in this study.

Optimal concentration of antibody used is essential to produce specific staining with minimal non-specific background staining in any application of immunohistochemistry. It is known that the rate of binding between antibody and antigen is affected by the affinity constant which in turn can be influenced by factors such as temperature, pH and buffer constituents. However, varying the relative concentrations of an antibody and an antigen can also influence the rate, extent and specificity of antibody-antigen complex formation (42, 51). Because it is impossible to change the antigen concentration, the optimal working concentration of each antibody must be determined with dilutions for each application and set for experimental conditions (42). New antibodies are usually received with recommended dilutions for various applications in the insert sheet; however, this can be the starting point as optimal staining for specific applications like this research can only be accomplished through a series of dilutions in a titration experiment. A titration experiment is done by first selecting a fixed incubation time and temperature then running a series of experimental dilutions of the antibody. It is also strongly recommended that each dilution should be tested on representative sample types to maintain the same experimental conditions that will be found with diagnostic samples.

Following optimization, the next step is validation to ensure consistency and reproducibility of the optimal staining protocol by testing the same optimal protocol on several different slides and specimen types with the expectation that close to optimal staining will be accomplished in each slide. It is common practice in immunohistochemistry especially in the diagnostic setting that the optimisation of the primary antibodies is followed by validation. This common practice also applies to validated antibodies that might be newly purchased from a different source or supply chain. Before these new validated antibodies are being introduced in the lab they must

be revalidated, and ideally the results must be compared to the previous lot. In this part of the research, the bulk of the bench work was concerned with optimizing and validating all the IHC protocols needed in later sections of the study. It is also important to state that this is the first study to optimise and validate Chromogranin A-Ki67 and Synaptophysin-Ki67 using concentrated antibodies. In contrast to the study of Matsukuma and colleagues who used ready to use (RTU) or pre-diluted antibodies from the manufacturer (9).

3.2 Methods

3.2.1 Case and control tissue selection

Three of the 20 eligible cases selected for this study were used for the optimisation and validation of the double immunohistochemistry stains of Synaptophysin-Ki67 and Chromogranin A-Ki67. The cases for optimisation were chosen to represent all the different specimen types used later in this research, a core biopsy, a resection specimen, and a gastric biopsy. There were no comparisons of the performance of each of the staining methods between specimen types conducted at this stage of the study.

A tonsil control block from our inhouse tissue bank was used for the optimisation of the Ki67-only stain. This was done primarily due to tonsil having a predictable high level of cell proliferation in B cells in the germinal centres. This guarantees the tissue will be positive for Ki67 antigen and that variation in staining intensity due to tissue fixation, antigen retrieval, antibody concentration and incubation time variables can be more easily assessed. A liver tissue control block was also used as the negative control because it is expected that fewer than 1% of the hepatocytes will be stained positive for Ki67 and no cytoplasmic staining.

The two markers Synaptophysin (Syn) and Chromogranin A (CgA) are known to react with neuroendocrine cells of the human adrenal medulla, pituitary, thyroid, lungs, pancreas, and gastrointestinal mucosa. Any of these tissue types can be used as a positive control tissue for both markers, however the pancreas offers more homogeneous and predictable staining making it a better choice for quality control. Because of this pancreas was preferred over other options for this study. A Syn/CgA/Ki67 control block was made from healthy tonsil and pancreas tissue and was added to each of the slides from the three cases used.

3.2.2 Preparation of tissues for IHC staining

A total of 20 sections were cut at 4 microns of thickness from the control block and 30 sections were cut from each of the 3 blocks (corresponding to each of the 3 selected cases) selected by the leading pathologist involved in this research. All were cut at 4 microns including the core biopsies. These sections were then transferred onto glass slides and were baked in a 60°C oven for 30 minutes. Each of the slides allocated to the double staining experiment also had a positive control added onto them. The positive control block was made up of a normal tonsil tissue and normal pancreas. The negative control which was only used in the Ki67-only slides was made up of liver tissue. The baking step was utilized to help prevent the sections from being washed

off during the staining process and to also aid in the removal of paraffin wax from the tissue sections. Following this step, slides were then allocated to their staining stations to commence staining. The staining of the immunohistochemistry slides was performed in the Leica BOND III IHC staining machine.

3.2.3 Immunohistochemistry Staining (Optimisation experiment)

3.2.3.1 The Leica Automated Bond III Platform

All IHC staining in this study was performed on the Leica Automated Bond III machines. This was to simplify the approach and to make the study results more directly transferable if an improvement in diagnostic outcome was identified and adopted. Most of the parameters (for example the antibody incubation temperature and secondary antibody dilution) have been optimised by Leica and adopted into all of the BOND III staining protocols (Table 3.3). The Bond III IHC machine is equipped with dewax solutions, an alcohol station, a wash buffer solution, a distilled water station, and two aqueous antigen retrieval solution options (ER1 and ER2), a bulk waste stations and a DAB waste station. Other reagents that are used in the Bond III machines includes the primary antibody (Ki67), a bond refine detection kit (contains a peroxide block, post primary, polymer reagent, DAB chromogen and haematoxylin counterstain), and a multi-detection kit (chromoplex kit) which house the red chromogen.

3.2.3.2 Primary Antibody

Three primary antibodies used in this study were: a DAKO mouse monoclonal antibody Ki67 (Clone MIB-1, Agilent Technologies Denmark); CELL MARQUE rabbit monoclonal antibody Synaptophysin (Clone MRQ-40, USA); and an INVITROGEN rabbit monoclonal antibody Chromogranin A (Clone 2N5Q4, China). Concentrated antibodies were preferred over ready-to-use antibodies as they offered more flexibility and potential cost savings especially in a large / high volume workload laboratory like Middlemore Hospital Histology Lab.

The DAKO mouse monoclonal antibody Ki67 clone MIB-1 had a manufacturer recommended dilution range of 1:75 to 1:150 (Appendix 2) as a starting point for optimisation. A dilution series was created around this range with 2-fold increments as suggested by the literature. The optimisation dilution series were 1:50, 1:75, 1:100, 1:150 and 1:200 (Table 3.1, below). Each dilution was made with x amount of the primary antibody Ki67 and y-amount of the BOND primary antibody diluent to make a total working volume of 1000 microlitre. i.e. antibody

dilution 1:100 =10 microlitre of the antibody Ki67 plus 990 microlitre of Bond primary antibody diluent to make up a total working volume of 1000 microlitre. Each slide needed 150 µL of Ki67 therefore it was important that the vial contained enough working solution throughout the experiment.

Table 3. 1. Antibody dilutions series for optimisation of the Ki67-only staining method.

Primary Antibody	**Antibody dilution**				
Ki67	1:50	1:75	1:100	1:150	1:200

The two rabbit monoclonal antibodies CELL MARQUE Synaptophysin clone MRQ-40 and the INVITROGEN clone 2N5Q40 Chromogranin A are used diagnostically as cytoplasmic markers of neuroendocrine tumours. Synaptophysin has a manufacturer recommended dilution range from 1:100 to 1:500 (Appendix 3). Chromogranin A on the other hand has a recommended dilution range from 1:50 to 1:200 (Appendix 4). Due to the high cost of antibodies and detection reagents required for this research, the antibodies of the double stain cocktails (i.e. CgA and Ki67) were optimized together. This means that both antibodies were diluted together in the same diluent, to provide different final concentrations for each. This also meant that the test dilution range was slightly different from that suggested by the manufacturer. (Table 3.2, below) (9). The manufacturer recommended dilution range was only used as a guide for this optimisation experiment.

Table 3. 2. Antibody dilutions series for optimisation of the proposed double staining methods (Syn-Ki67 and CgA-Ki67).

Double stain	**Antibody Dilutions**					
Syn-Ki67 (1:100-1:500 / 1:75-1:150)	1:50 / 1:50	1:100 / 1:100	1:200 / 1:200	1:500 / 1:500	1:700 / 1:700	
CgA-Ki67 (1:50-1:200/ 1:75 -1:150)	1:100 / 1:100	1:100 / 1:75	1:500 / 1:50	1:1000 / 1:50	1:4000 / 1:50	1:6000 / 1:50

3.2.3.3 Antigen Retrieval

Antigen retrieval in the optimisation experiments of the Ki67 and the two double staining methods (Synaptophysin-Ki67 and Chromogranin A-Ki67) was done using the heat induced epitope retrieval (HIER) method. This involved incubating FFPE tissue sections in either acidic or alkali buffer solution at a high temperature of 100 degrees Celsius for 20 minutes. The Leica Bond III machine have two forms of aqueous antigen retrieval solutions to choose from: Epitope Retrieval 1 (ER1) and Epitope Retrieval 2 (ER2). ER1 is a ready to use (RTU) citrate-based epitope retrieval solution (pH 6) for HIER in the Bond III system. ER2 is a ready to use (RTU) antigen retrieval solution that contains tris EDTA and surfactant (pH9) (43). For each of the staining methods tested in this study, various antigen retrieval protocols (combination of antigen retrieval solutions and time) were trialled to determine the optimal antigen retrieval method.

3.2.3.4 Optimisation of the Ki67-only stain.

The optimisation of the Ki67-only stain was designed to test various staining protocols to find the optimal staining procedure for Ki67. Outlined below in Table 3.4, are different procedures that were trialled in which each slide comprised of a section of tonsil and liver and were stained with different antibody dilutions, epitope retrieval (antigen retrieval) and buffer solutions at different antigen retrieval times.

Table 3. 3. Bond III IHC Staining Protocol for the Ki67-only stain.

Step	Reagent	Time (min)	Temperature (°C)
1	Bond Dewax Solution x 3 changes	30	72
2	Alcohol x 3 changes	0	RT
3	Bond wash solution x 3 changes	0	RT
4	Bond ER Solution (ER1 or ER2)	20 or 30	100
5	Bond wash solution x 5 changes	3	RT
6	Primary antibody anti-Ki67 Various concentration or antibody dilution: 1:50, 1:75, 1:100, 1:150, 1:200	15	RT
7	Bond wash solution x 3 changes	0	RT
8	Post-primary	8	RT
9	Bond wash solution x 3 changes	6	RT
10	Polymer	8	RT
11	Bond wash solution x 2 changes	4	RT
12	DI water wash	0	RT
13	Peroxide block	5	RT
14	Bond wash solution x 3 changes	0	RT
15	Mixed DAB refines detection kit	10	RT
16	DI water wash x 3	0	RT
17	Haematoxylin	7	RT
18	DI water wash	0	RT
19	Bond wash solution x 1 change	0	RT
20	DI water wash	0	RT

RT- Room Temperature. Highlighted in yellow are the variables that were optimised. Other parameters including the incubation time (of 15 mins) of the primary antibody were standardized. The other highlighted steps in the protocol are significant steps in the staining protocol i.e. the addition of the peroxide block in step 13. 0 min means instant or less than a minute.

Following preparation of tissues, slides were loaded into the Bond III machines, where tissue sections were dewaxed further in dewaxing solutions for 30 minutes at 72 °C (Step 1, Table 3.3), alcohol/buffer washed, then underwent heat-induced antigen retrieval (HIER) at 100 °C in various aqueous antigen retrieval (buffer) solutions and various antigen retrieval times (Table 3.3&3.4). Following antigen retrieval, the sections were then washed in 5 changes of buffer wash solution. After a quick wash of the section, it was then incubated in 150 µL of post primary IgG linker (Highlighted in green, Step 8, Table 3.3 above). The linker is a rabbit anti-mouse IgG that binds to the primary MIB-1 Ki67 monoclonal antibody. Unbound linker was washed off in three changes of buffer wash solution for six minutes. The section was then incubated with a polymer anti-rabbit poly-HRP IgG reagent which localises rabbit antibodies. Unbound polymer was washed off in two changes of buffer wash for 4 minutes then rinsed in DI water. The section was then incubated in 3% hydrogen peroxide for 5 minutes to block

endogenous peroxidase activity in the section. The tissue section was then quickly washed with buffer wash before being incubated with 150 µL of 3,3-Diaminobenzidine tetrahydrochloride (DAB) solution for 10 minutes then washed with deionised water. The sections were then counterstained with haematoxylin.

Once staining on the BOND III machine was completed, slides were then unloaded and underwent dehydration in a series of graded alcohol, cleared in xylene, mounted and cover slipped using the Leica CV5030 Fully Automated Cover Slipper. The now stained sections were then quality checked for positive staining and several other QC parameters that includes the following: depth of stain/ colour intensity, distribution of positive areas within lymph node, germinal centres of tonsils should be darker with high proportion of cells staining, distribution of DAB to the nuclei only, ability to see the counterstained nucleus in addition to the DAB stain, intensity of the overall counterstain and lack of non-specific staining. They were also compared to inhouse controls. Once the optimal staining protocol was determined, it was then further evaluated for consistency and reproducibility by performing validation runs on a further 5 sections from different tonsil control block considering the QC parameters listed above (Please refer to validation table, Table 3.8 below).

Table 3. 4. Summary of the optimisation and experimental design for Ki67-only stain.

Antibody Dilutions	No. of Trial					
	Trial 1		Trial 2		Trial 3	
	ER1(20min)	ER2(20min)	ER1(30min)	ER2(30min)	ER2(20min)	ER2(30min)
1:50	☒	☒	☐	☐	☐	☐
1:75	☒	☒	☐	☐	☐	☐
1:100	☒	☒	☒	☒	☒	☒
1:150	☒	☒	☒	☒	☐	☐
1:200	☒	☒	☒	☒	☒	☒

***Key**: ER1 and ER2 are antigen retrieval solution and the numbers inside the brackets are HIER treatment times in minutes. HIER temperature- 100 degrees Celsius.*

Table 3.4 above shows the stepwise and trial-based strategy to determine the optimal staining protocol for the Ki67-only staining method. The three trials undertaken read from left to right and were performed sequentially in time.

3.2.3.5 Optimisation of the dual staining protocol (Syn-Ki67 and CgA-Ki67)

The pre-optimisation of the double staining protocols followed similar procedures to that of the Ki67-only staining method. 4 µm sections from three of the study eligible cases were mounted onto glass slides together with a section of the inhouse pancreas/tonsil control.

Outlined in Tables 3.5-3.7 below, are the procedures that were strictly followed to determine the optimal staining protocol for each of the double staining methods tested in our study. The dilution range suggested by the manufacturer for Synaptophysin is 1:100 to 1:500 and 1:50 to 1:200 for Chromogranin A. Antibody dilution series for each of the proposed double staining protocols were created around the manufacturer dilution ranges in 2 or 3-fold increments as suggested in the literature with various antigen retrieval methods.

Synaptophysin-Ki67 AB Dilution Series 1:50/ 1:50, 1:100/ 1:100, 1:200/ 1:200, 1:500/ 1:500, and 1:700/ 1:700.

Chromogranin-A- Ki67 AB Dilution Series 1:100/ 1:100; 1:100/ 1:50; 1:200/ 1:50; 1:500/ 1:1:50; 1:1000/ 1:50; 1:4000/ 1:50 and 1:6000/ 1:50

Table 3. 5. BOND III IHC Staining Protocols for the Proposed Dual Staining Methods Syn-Ki67 and CgA-Ki67.

Step	Reagent	Time (min)	Temperature (°C)
1	Bond Dewax Solution x 3 changes	30	72
2	Alcohol x 3 changes	0	RT
3	Bond wash solution x 3 changes	0	RT
4	Bond ER Solution (ER1 or ER2)	AR (20, 30 or 40)	100
5	Bond wash solution x 5 changes	3	RT
6	Peroxide Block	5	RT
7	Bond wash solution x 3 changes	0	RT
8A **8B**	Cocktail A or Cocktail B	15	RT
9	Bond wash solution x 3 changes	0	RT
10	Polymer mHRP	8	RT
11	Bond wash solution x 3 changes	0	RT
12	Polymer rAP	20	RT
13	Bond wash solution x 5 changes	5	RT
14	DI water	0	RT
15	Mixed DAB refine detection kit	10	RT
16	DI water x 3 changes	0	RT
17	Red chromogen added	15	RT
18	DI water x 3 changes	0	RT
19	Haematoxylin	5	RT
20	DI water	0	RT
21	Bond wash solution x 1 wash	0	RT
22	DI water	0	RT

KEY: AR Time= Antigen Retrieval Time, RT=Room Temperature

Cocktail A: cocktail of the Synaptophysin-Ki67 which have a serial antibody dilution of 1:50, 1:100, 1:200, 1:500, and 1:700

Cocktail B: cocktail of the Chromogranin A-Ki67 with a series of antibody dilution 1:100/ 1:100; 1:100/ 1:50; 1:200/ 1:50; 1:500/ 1:1:50; 1:1000/ 1:50; 1:4000/ 1:50 and 1:6000/ 1:50.

Highlighted in yellow are the variables that were changed or optimised throughout the optimisation process to determine the optimal staining protocol for the proposed staining methods. Other highlighted (green) steps are significant steps in the staining process. Parameters such as antibody and polymer incubation time, antigen retrieval incubation temperature of 100 °C and DAB detection were kept constant throughout. Brown DAB refining kit (blue) and the mixed red refining kit (red) were applied sequentially.

As shown in Table 3.5 above, the two proposed double staining methods used the same BOND III IHC staining protocols. The only step that changed was Step 8 where the cocktails used were either Cocktail A (Syn-Ki67) or Cocktail B (CgA-Ki67). The rest of the protocol was the same. After 30 minutes of slide heating in the 60-degree Celsius oven, sections were dewaxed further in bond dewaxing solution for 30 minutes at 72 °C, washed in three changes of alcohol and buffer washing solution, then underwent heat antigen retrieval (HIER). HIER was conducted at 100 °C in one of the two epitope retrieval solutions using various antigen retrieval

times (e.g.20, 30 or 40 min) (Table 3.6, below). Sections were then washed with buffer washing solution for 3 minutes and then incubated in 3% of hydrogen peroxide for 5 minutes. The hydrogen peroxide was then washed off before the addition of the antibody cocktails (Syn-Ki67 or CgA-Ki67) at various antibody dilutions (Table 3.6 (Syn-Ki67) &3.7 (CgA-Ki67)) and incubated for 15 minutes. The slide was then quickly washed in 3 changes of buffer washing reagent before the addition of the goat anti-mouse IgG micropolymer labelled with HRP. This secondary antibody binds to the MIB-1 Ki67 mouse monoclonal antibody which is already bound to Ki67 antigens in the nuclei. This was followed by another 3 changes of buffer wash to remove unbound antibody and then the Polymer rAP was added. This is the second secondary antibody which is an alkaline phosphatase (AP) labelled **goat anti-rabbit IgG micro-polymer**. Following these steps, the section was then washed in another five changes of buffer wash, rinsed in distilled water before the addition of the brown and red chromogen. After the sequential addition of the two contrasting chromogens, slides were then washed in three changes of distilled water, counterstained in haematoxylin, before a three step wash off. The slides were then dehydrated in two changes of alcohol, cleared in xylenes, before cover slipping using a xylene based mounting medium, ready for evaluation.

Table 3. 6. Summary of Optimisation and experimental design for the Syn-Ki67 DS method.

Antibody Dilution	No. of Trials				
	Trial 1		Trial 2		Trial 3
	ER1(20mins)	ER2(20mins)	ER2(30mins)	ER2(40mins)	ER2(20mins)
1:50/ 1:50	☐	☒	☒	☒	☒
1:100/ 1:100	☒	☒	☐	☐	☐
1:200/ 1:200	☒	☒	☐	☐	☐
1:500/ 1:500	☒	☒	☐	☐	☐
1:700/ 1:700	☒	☒	☐	☐	☐

***Key**: ER1 and ER2 are antigen retrieval solution and the numbers inside the brackets are HIER treatment time in minutes. The HIER temperature was set to 100 degrees Celsius for all the optimisation experiments in this study.*

Presented in Table 3.6 above is the experimental design and summary of the strategic method used economically to optimise the Synaptophysin-Ki67 double staining method. A total volume of 1 mL (1000 µL) was made for each of the antibody dilution series. This was done to ensure enough working solutions for the entire optimisation/validation experiment as 150 µL is required for each test on the Bond III IHC platform.

Table 3. 7. Summary of the optimisation and experimental design for the CgA-Ki67 DS method.

Antibody Dilutions	No. of Trials					
	Pre-trial		Trial 1		Trial 2	
	ER1 (20min)	ER2 (20 min)	ER1 (20 min)	ER2 (20 min)	ER2 (20 min)	ER2 (30 min)
1:100/1:100	☒	☒	☐	☐	☐	☐
1:100/ 1:50	☒	☒	☐	☐	☐	☐
1:200/ 1:50	☐	☐	☒	☒	☐	☐
1:500/ 1:50	☐	☐	☒	☒	☐	☐
1:1000/1:50	☐	☐	☒	☒	☐	☐
1:4000/1:50	☐	☐	☒	☒	☒	☒
1:6000/1:50	☐	☐	☒	☒	☒	☒

***Key**: ER1 and ER2 are antigen retrieval solution and the numbers inside the brackets are HIER treatment time in minutes. The HIER time was set to 100 degrees Celsius for all the optimisation experiments in this study.*

A preliminary trial was used in the optimisation experiment of the Chromogranin A-Ki67 double staining method. This was done to help define the direction to take in terms of optimising the methods (e.g. a starting point for antibody dilution) in trial. In the preliminary trials as shown in Table 3.7 above, the tissue sections were incubated with 150 µL of each of the 1:100/ 1:100 Chromogranin A-Ki67 cocktail and 1:100/ 1:50 Chromogranin A-Ki67 cocktail with varies antigen retrieval methods (ER1 (20mins) or ER2 (20mins)). Based on the outcome of the preliminary trial, we then used the antibody dilution range for the chromogranin-A as suggested by the manufacturer (1:50 to 1:200, Appendix B) to create a dilution series that started at 1:200 and went all the way up to 1:6000 while keeping the antibody dilution of Ki67 constant at 1:50. There were only two attempts made in the optimisation experiment of the Chromogranin A-Ki67 staining to determine its optimal staining protocol.

3.2.4 Validation of each method for reproducibility and consistency.

Table 3. 8. Validation Table for all three IHC staining methods.

Staining Method/ Validation No.	Study ID/Case ID/ Tissue type of control used	Validation1	Validation 2	Validation 3	Validation 4	Validation 5
Ki67-only stain	Tonsil	☑	☑	☑	☑	☑
Double stain Syn-Ki67	Case 1 19/P21340.1B	☑	☑	☑	☑	☑
	Case 10 23/P22529.1D	☑	☑	☑	☑	☑
	Case 5 22/P26103.1A	☑	☑	☑	☑	☑
Double stain CgA-Ki67	Case 1 19/P21340.1B	☑	☑	☑	☑	☑
	Case 10 23/P22529.1D	☑	☑	☑	☑	☑
	Case 5 22/P26103.1A	☑	☑	☑	☑	☑

***Note**: In this validation table, whenever a test is passed, we ticked the box.*

In this part of the study, each of the three staining methods (Ki67-only stain, Syn-Ki67 and the CgA-Ki67) were validated for consistency and reproducibility by running the optimal staining protocols determined in the optimisation of antibodies for each method:

- **Ki67-only stain**- antibody dilution of 1:100 with antigen retrieval method: ER2 in 30 minutes.
- **Synaptophysin-Ki67 DS stain**- antibody dilution of 1:50 (Syn) /1:50 (Ki67) with antigen retrieval method: ER2 in 20 minutes.
- **Chromogranin A-Ki67 DS stain**- antibody dilution of 1:6000 (CgA)/ 1:50 (Ki67) with antigen retrieval method: ER2 in 20 minutes.

Five slides of tonsil control tissues were used for the validation trials of the optimised Ki67-only staining method. Alongside the section of tonsil was a liver section that was used as a negative control. There were also five slides each of the selected cases (resection or biopsy) with appropriate controls (pancreas and tonsil tissues) used in the validation trials of the double staining methods (Syn-Ki67 and CgA-Ki67). This would mean a total of 15 slides for the validation of the Synaptophysin-Ki67 DS staining method and 15 slides for the validation of the Chromogranin A-Ki67 DS staining method. Each validation slides were quality checked according to the following quality control criteria; depth of stain/ colour intensity, distribution of positive areas within lymph node, germinal centres of tonsils should be darker with high

proportion of cells staining, distribution of DAB to the nuclei only, ability to see the counterstained nucleus in addition to the DAB stain, intensity of the overall counterstain and lack of non-specific staining. Whenever a validation test was passed or successful the corresponding box for the test on the table above (Table 3.8) was marked with a tick. Further validation for reproducibility and consistency was conducted in the next part of the research using a total of 20 eligible cases.

3.2.5 Evaluation of Staining Quality

Single IHC Staining method (Ki67-only stain) targeting Ki67antigen in tonsil.

The quality of the stains was quality checked using the Olympus BX53 microscope for positivity, specification, intensity of the stain and present of background staining. As stated previously, a qualitative scoring method (48-50) was adopted in this study for the scoring of the staining intensity. With this scoring system, the quality of the stain were evaluated and scored as negative (-), weak positive stain (+), moderate (++) and strong positive stain (+++) (47).

Dual stain targeting Ki67 and the marker (Synaptophysin and Chromogranin A) for NET tissue together.

The quality of the stains for the double stains were also evaluated using a qualitative scoring system (48-50, 58). The Ki67 in the double stain was expected to be strong positive (+++), and the cytoplasmic markers Synaptophysin to be strong positive (+++) and Chromogranin A to be moderate positive (++). This balance in staining intensities between the markers were required for both markers to be expressed and none masking the other. In each of the trial, sections were quality checked under the microscope for positivity, specificity and intensity of both cytoplasmic and nuclear stains.

3.3 Results

3.3.1 Ki67-only staining method

The intensity of nuclear staining with DAB varied as a function of antibody concentration, antigen retrieval buffer selection and time. Homogeneously stained nuclei predominated over a punctuate stain pattern and the colour spectrum was from mid brown to dark brown. When the staining evaluation criteria were applied weak staining (+) implied that the chromatin counterstain (blue) pattern was visible together with pale brown. Moderate (++) and strong (+++) staining was consistent with the blue chromatin colour being largely or fully obscured by mid to dark brown DAB tones. A Ki67 stain result was considered too strong if the nuclei were dark brown to black, if the volume and intensity exaggerated the expected boundary of the nucleus and cell and if there was non-specific staining, i.e. extra-cellular matrix or the cytoplasm of non-target cells, for example liver (Fig 3.3). The preferred staining protocol or optimal staining protocol for the Ki67-only staining method was **antibody dilution 1:100, antigen retrieval buffer solution ER2 and an antigen retrieval time of 30 minutes**. This staining protocol successfully demonstrated the Ki67 antigen in the tonsil germinal centres (Figure 3.1 below). The nuclei of B cells in the germinal centres were not overly stained and there was no non-specific background staining. The other staining protocols trialled were eliminated as they did not produce the optimal staining quality preferred. This is shown in the flow diagram (Figure 3.2) below.

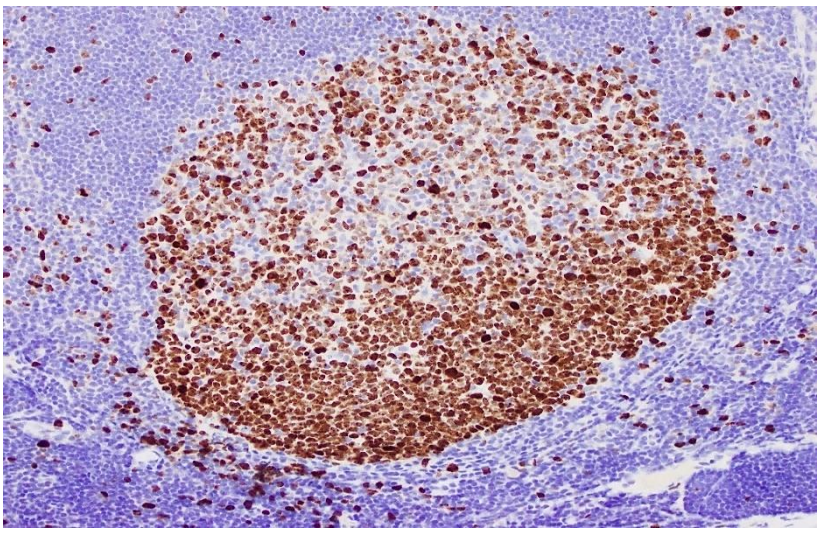

Figure 3. 1. Optimal staining for Ki67-only.

Magnification 400x. Shown here is the germinal centre of a normal tonsil stained with the optimal staining protocol for the Ki67-only staining method: ***antibody dilution 1:100, antigen retrieval buffer solution ER2 and an antigen retrieval time of 30 minutes.***

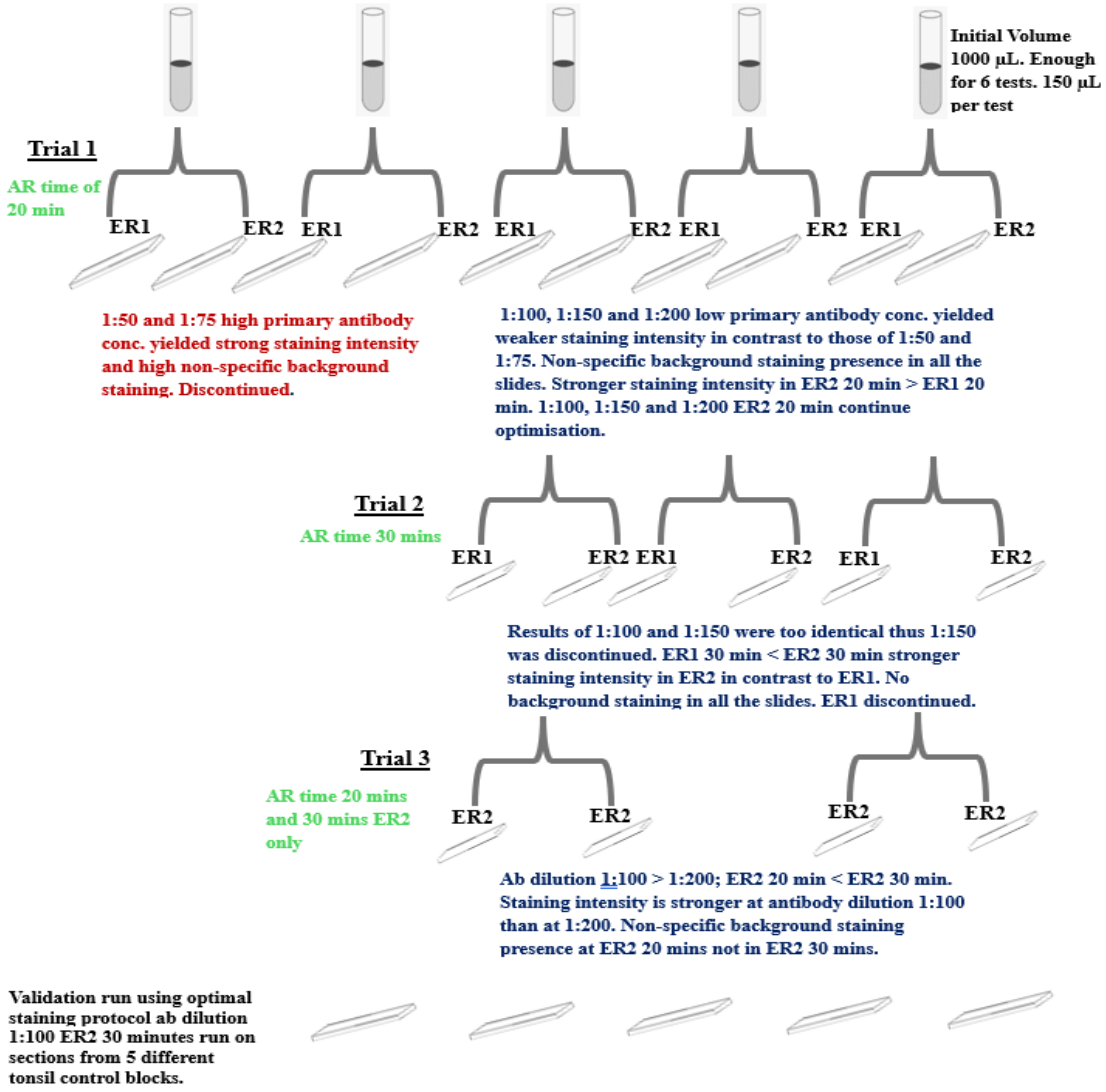

Figure 3. 2. Flow Diagram of the optimisation and validation of the Ki67-only.

Ki67 antibody concentration is given as the dilution of its stock (Appendix 2). AR- Antigen retrieval time (minutes) using epitope retrieval solution 1 (ER1) or epitope retrieval solution 2 (ER2). Serial antibody dilution: 1:50, 1:75, 1:100, 1:150, and 1:200.

The flow diagram (Figure 3.2) above showcases the processes taken to successfully determine the optimal staining protocol for the Ki67-only staining method. It was separate from the dual staining procedure and demonstrates the logic of how variables such as time of antigen retrieval were adjusted to achieve optimal staining quality. Apart from the concentration of the primary antibody (Ki67) and the antigen retrieval method, all the other parameters i.e. antibody incubation time in the staining protocol for Ki67 were standardised or remained constant throughout the experiment. This means that the only variables or parameters that changed

throughout were the concentration of the primary antibody (Ki67) also called antibody dilution, the type of antigen retrieval (ER) solution used and antigen retrieval time.

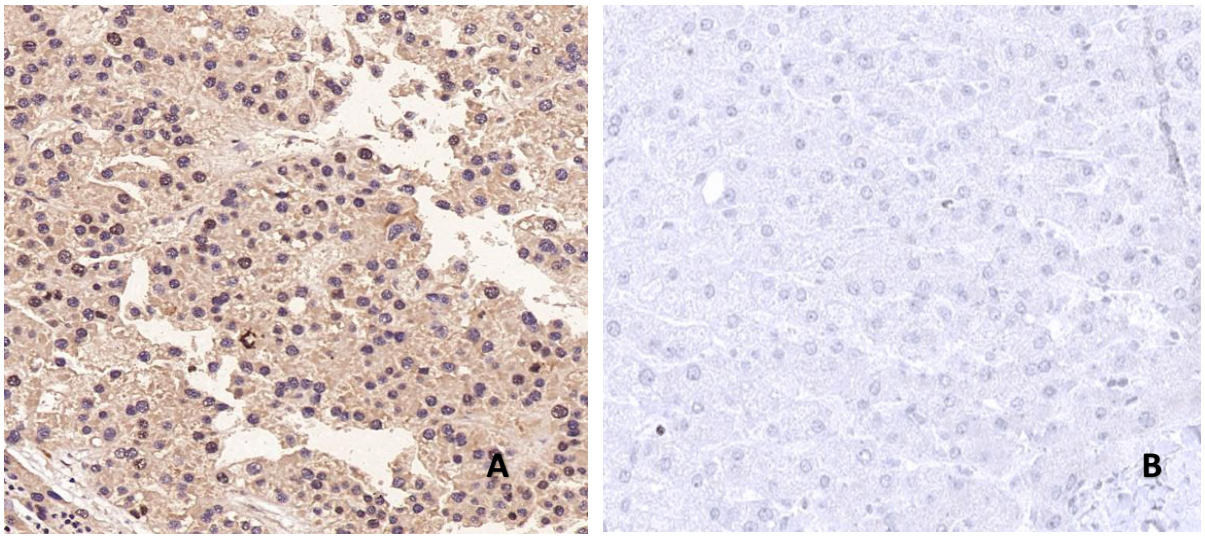

Figure 3. 3. Comparative images of Liver Tissue Negative Control when stained with different concentrations of the Ki67 antibody.

Magnification x200. Diagram A: Non-specific staining on the liver tissue negative control when concentration of the Ki67 was high. Diagram B: Negative control stained with the optimal staining protocol. Less than 1 percent of the hepatocytes stained and no cytoplasmic staining.

Following the first trial (Experiment trial no.1), antibody dilutions 1:50 and 1:75 were eliminated due to intensities of the stains being too strong with high non-specific background staining (Figure 3.2 and Table 3.9 below). Non-specific background staining was also seen in the liver tissue section used as the negative control on these slides (Figure 3.3, Panel A). From this trial (Trial no.1), low antibody dilutions 1:100, 1:150 and 1:200 also had background staining although yielded weaker staining intensity in contrast to the ladder. The staining intensity produced by ER1 20 mins was much weaker when comparing to those of ER2 20 mins. When the antigen retrieval time was increased from 20 to 30 minutes in Experimental Trial no. 2, the staining intensities in ab dilutions 1:100, 1:150 and 1:200 became a lot stronger than they were when the antigen retrieval time was 20 minutes in Experimental Trial no.1. No non-specific background staining seen in Trial no.2 at ER2 30 minutes (Figure 3.4 below). The results yielded by ab dilution 1:100 and 1:150 in Trial no.2 were too similar thus 1:150 was discontinued as well as the ER1 solution. Based on the outcome of the second trial we further optimised the antibody Ki67 by testing the antibody dilutions 1:100 and 1:200 with just the antigen retrieval solution ER2 at 30 minutes retrieval time. This attempt yielded very similar staining quality between the two staining protocols (antibody dilutions 1:100 ER2 30 mins vs

1:200 ER2 30 mins) but a much preferred and stronger staining power when antibody dilution was 1:100 (Figure 3.1 and Table 3.9 below). The liver tissue section that was used as negative control on this slide also presented no non-specific background staining (Figure 3.3, Diagram B above).

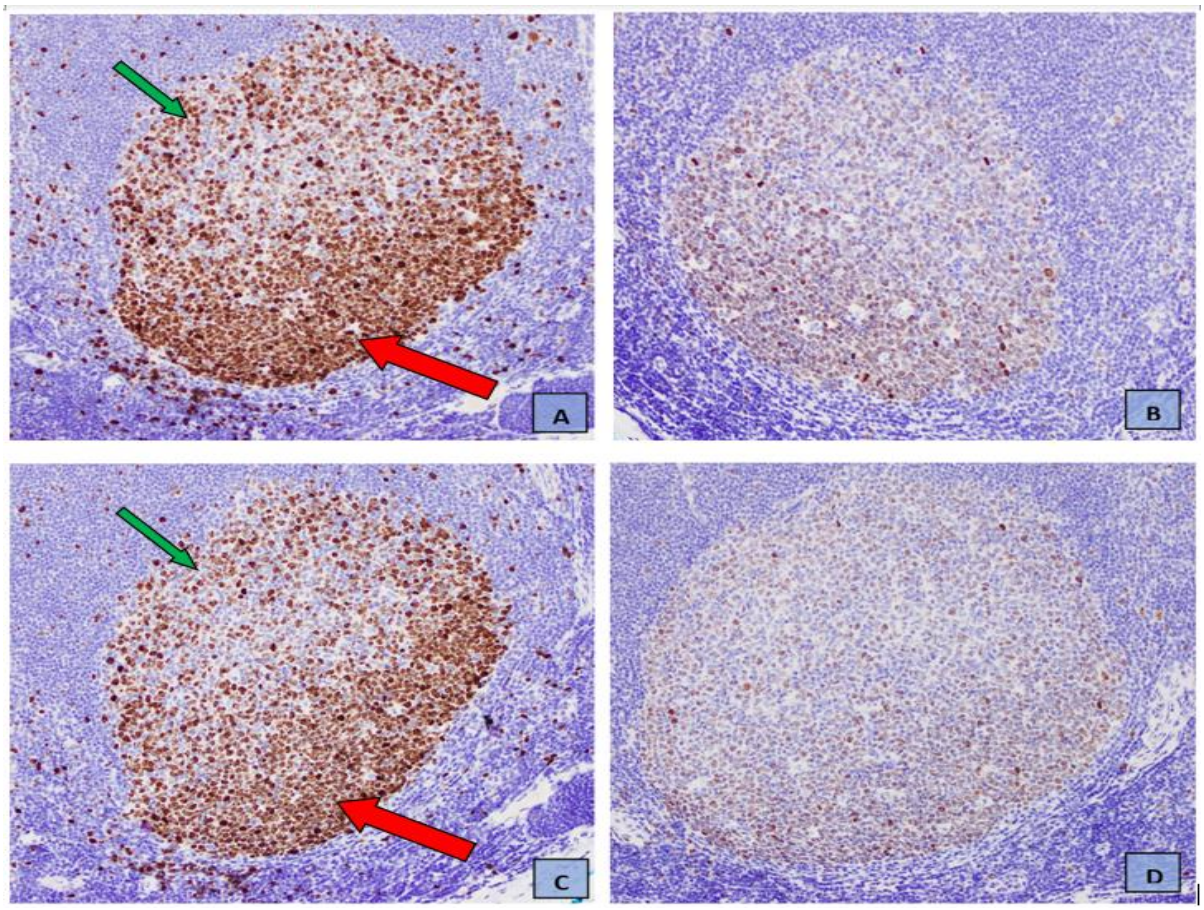

Figure 3. 4. Staining patterns on germinal centres of a normal tonsil at different staining protocols.

Magnification 400x. Shown here are sections from a healthy normal tonsil with increased proliferation (Ki67 staining) in the germinal centres and lower staining in the surrounding mature cells. Proliferating cells labelled with the gold standard Ki67 marker are concentrated in the dark zone of the germinal centre (Red Arrow) away from site of antigen entry from the tonsillar surface (Green Arrow) ***A)*** *Germinal centre showing strong positivity for Ki67 Ab dilution 1:100 ER2: 30 min* ***B)*** *Germinal centre showing weak staining for Ki67 Ab dilution 1:100 ER1: 30 mins* ***C)*** *Shown here is a strong positivity for Ki67 in the germinal centre Ab dilution 1:200 ER2:30 mins* ***D)*** *Weak expression for Ki67 in the germinal centre due to Ab dilution 1:200 ER1:20 mins.*

Table 3. 9. Result from the optimisation of the Ki67-only staining method.

No. of Trial	Antibody Dilution	Antigen Retrieval Method ER1 or ER2 (time in minutes)	Sensitivity of Stain	Background stain
Trial 1	1:50	ER1(20)	Too strong	High background stain
		ER2(20)		
	1:75	ER1(20)		
		ER2(20)		
	1:100	ER1(20)	+	Some
		ER2(20)	++	
	1:150	ER1(20)	+	Some
		ER2(20)	+	
	1:200	ER1(20)	-	Some
		ER2(20)	+	
Trial 2	1:100	ER1(30)	++	Some
		ER2(30)	+++	None
	1:150	ER1(30)	+++	None
		ER2(30)	++	None
	1:200	ER1(30)	+	None
		ER2(30)	++	
Trial 3	1:100	ER2(20)	++	None
		ER2(30)	+++	None
	1:200	ER2(20)	+	None
		ER2(30)	++	

Key: *Qualitative Score: (-) negative stain (+) weak positive stain (++) moderate positive stain (+++) strong positive stain*

3.3.2 Synaptophysin-Ki67 Double Stain.

The distribution, intensity and specificity of the synaptophysin stain was of primary interest due to prior knowledge about the behaviour of Ki67. Synaptophysin was confined to the cytoplasm of the tumour cell population and cells with a neuroendocrine phenotype. The counterstained (blue) nuclei were routinely visible. Synaptophysin staining was characterized as both a homogeneous pink blush and containing punctate crimson elements. The Syn stain was considered too strong at some lower recommended dilutions when a homogeneous, intense crimson colour dominated the tumour cell population. Optimal staining intensity factored the need to clearly identify and count proliferating cells stained with Ki67 and to clearly which cells stain both with Syn and Ki67. Proliferating cells presented with a brown stained nucleus. The character of Ki67 was mostly homogeneous and in the mid to dark brown tone range. The qualitative scoring criteria were applied to scoring intensity, noting the nuclei of neuroendocrine cells as larger than lymphocyte nuclei (Table 3.10 below). The optimal staining protocol for the Synaptophysin-Ki67 double stain was**: antibody dilution Syn 1:50/ Ki67 1:50 with antigen retrieval method (antigen retrieval solution ER2 and antigen retrieval times of 20 mins)** With this protocol, the Synaptophysin-Ki67 double stains successfully demonstrated Ki67 antigen, in the DAB brown spectrum and confined to the nucleus, as well as Synaptophysin, distinct and contrasting crimson red, in the cytoplasm of the tumour cell population (Figure 3.5, below).

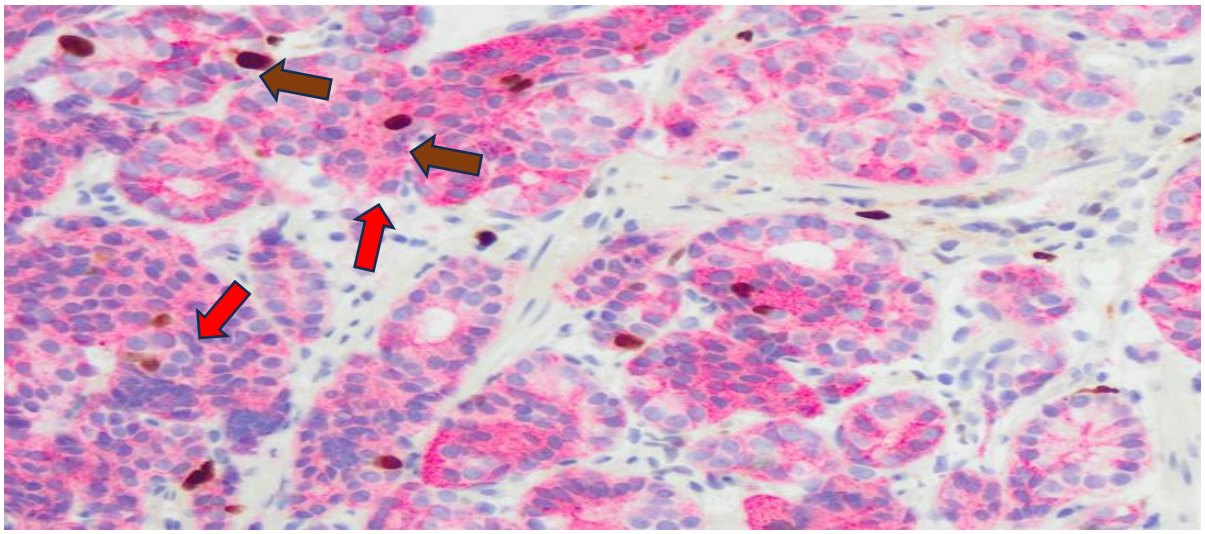

Figure 3. 5. Synaptophysin-Ki67 Double Stains.

Magnification 200x. The red arrows are showing the Synaptophysin antigens found in the cytoplasm of the tumour cell population stained in distinct and contrasting red. The brown arrows are showing the Ki67 antigens localized in the nuclei of tumour cells.

The flow diagram below shows the strategic method used to determine the optimal staining protocol for the proposed Synaptophysin-Ki67 double staining method. All the antibody concentrations (expressed in antibody dilution series 1:100/ 1:100, 1:200/ 1:200, 1:500/ 1:500 and 1:700) lower than Syn 1:50/ Ki67 1:50 yielded relatively weak staining intensities regardless of the antigen retrieval solution and time used (Table 3.10 below).

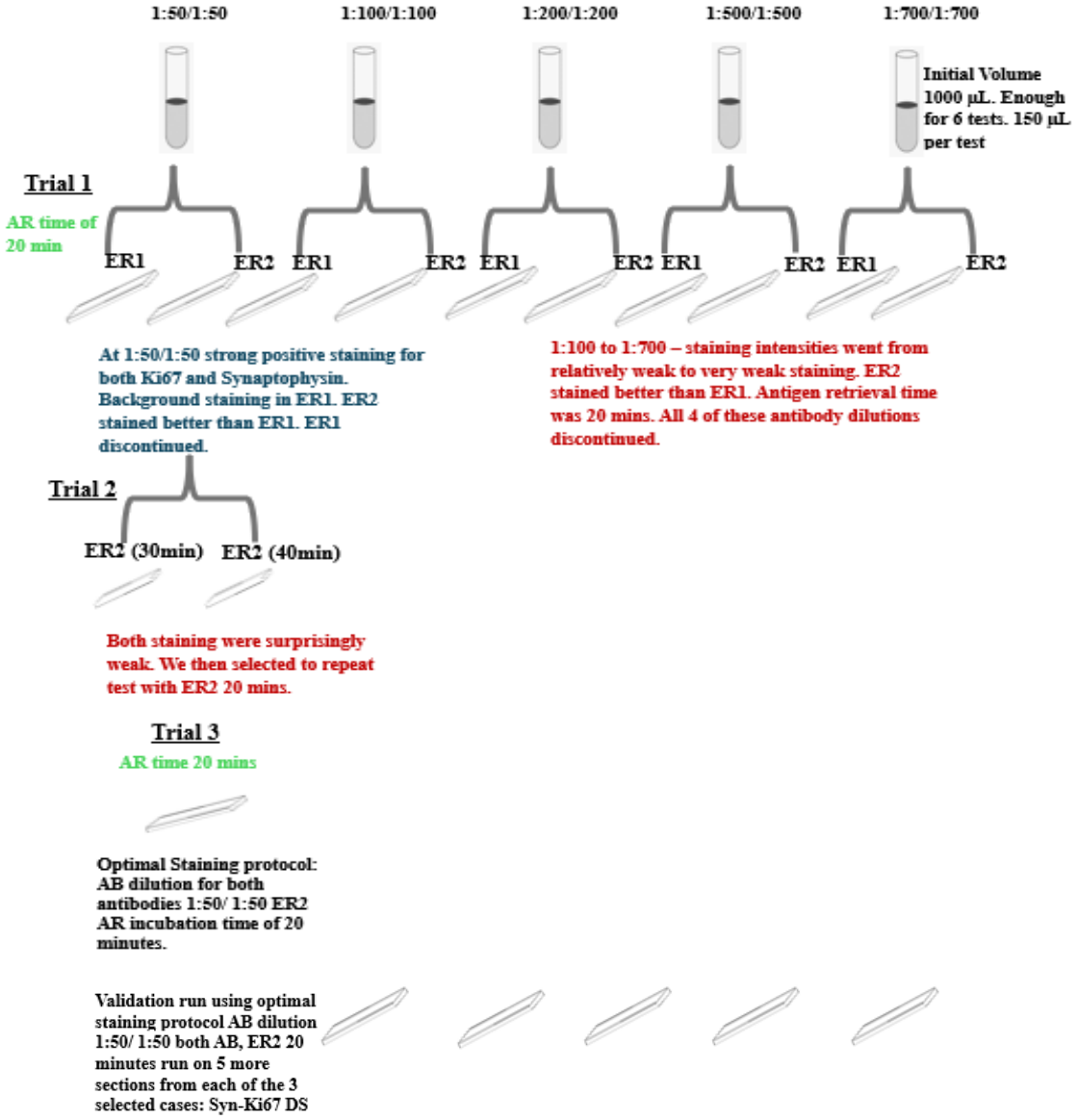

Figure 3. 6. Flow Diagram of the optimisation and validation of the Syn-Ki67 DS Method.

Antibody dilution series Syn-Ki67 1:100/1:100, 1:200/1:200, 1:500/1:500 and 1:700/1:700 was discontinued due to weak expressions of both cytoplasmic Synaptophysin and nuclear Ki67 antigens. Antibody dilution Syn-Ki657 1:50 was adjusted accordingly depends on quality of stains preferred.

As shown in Figure 3.6 above and the table below (Table 3.10), following the first experimental trial the focus was then shifted to optimising the double staining method using antibody dilution Syn-Ki67 1:50/ 1:50- ruling out the use of antigen retrieval solution ER1 due to background staining. In Trial no.2, when the antigen retrieval time were increased to 30 and 40 minutes the intensity of the staining at the tested ab dilution of Syn 1:50/ Ki67 1:50 became surprisingly weaker (Figure 3.7, Panel A and B below).

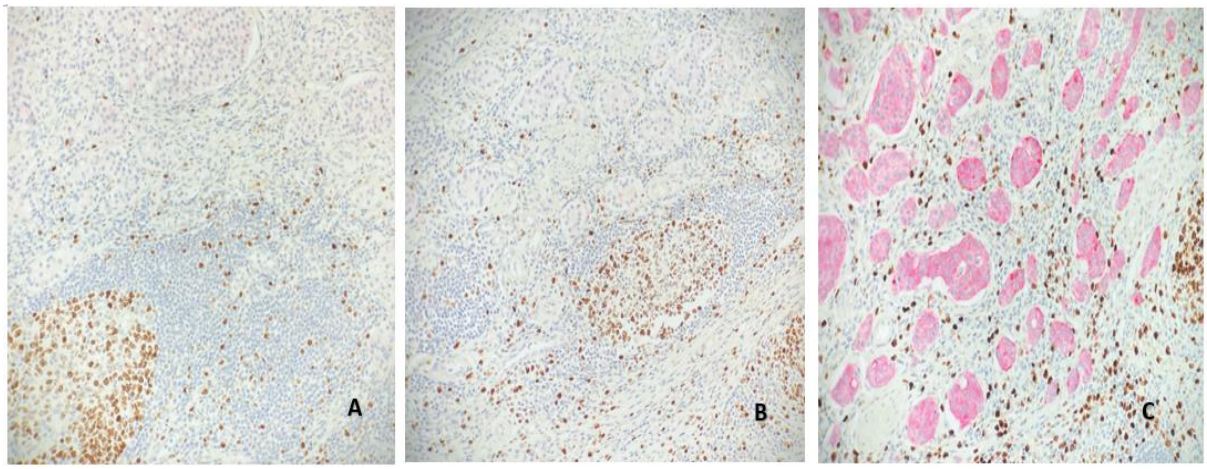

Figure 3. 7. Comparison of antigen retrieval time.

Magnification 100x. Showing above are comparative images of different staining qualities yielded between different antigen retrieval times (A-30 min vs B-40 min vs C-20 min) antibody dilution Syn-Ki67 1:50/1:50 antigen retrieval solution ER2. Images are from one of the three selected cases.

In Trial No.3, the staining protocol (*antibody dilution Syn-Ki67 and antigen retrieval solution ER2, antigen retrieval times of 20 mins*) in which tested parameters were repeated from Trial No.1, the intensity of the stain for both markers increased (Syn +++/ Ki67 +++) where the cytoplasmic markers synaptophysin appeared crimson red and the nuclear marker Ki67 appeared dark brown (Figure 3.7, Panel C above).

Table 3. 10. Result from the optimisation of the Syn-Ki67 DS method.

No. of Trial	Antibody dilution	Antigen Retrieval Method (ER1 or ER2 and time in minutes)	Sensitivity of Stain	Background stain
Trial 1	1:50/ 1:50	ER1(20)	+++ SYN + Ki67	Some
		ER2(20)	+++ SYN +++ Ki67	Some
	1:100/ 1:100	ER1(20)	++ SYN + Ki67	Some
		ER2(20)	++ SYN ++ Ki67	
	1:200/ 1:200	ER1(20)	+ SYN + Ki67	None
		ER2(20)	+ SYN - Ki67	
	1:500/ 1:500	ER1(20)	- SYN + Ki67	-
		ER2(20)	- SYN + Ki67	
	1:700/ 1:700	ER1(20)	- SYN + Ki67	-
		ER2(20)	- SYN + Ki67	
Trial 2	1:50/ 1:50	ER2(30)	- SYN ++ Ki67	Some
		ER2(40)	- SYN ++ Ki67	
Trial 3	1:50/ 1:50	ER2(20)	+++ SYN +++ Ki67	Some

***Key:* Qualitative Score: (-) negative stain (+) weak positive stain (++) moderate positive stain (+++) strong positive stain**

3.3.3 ChromograninA-Ki67 Double Stain

The character of the Chromogranin A stain, its distribution, colouration and intensity, was very similar to Synaptophysin and evaluated independently of Ki67. However, there are key differences in that chromogranin A tends to present a more granular staining pattern within the cytoplasm while synaptophysin exhibits a more diffuse staining pattern throughout the cytoplasm. The preferred staining protocol for the Chromogranin A-Ki67 double staining method was **antibody dilution CgA 1:6000/ Ki67 1:50, antigen retrieval buffer solution ER2 and an antigen retrieval time of 30 minutes**. Like the Synaptophysin-Ki67 staining method presented above, this protocol also successfully demonstrated Ki67 antigen, in the DAB brown spectrum and confined to the nucleus, as well as Chromogranin A, distinct and contrasting red, in the cytoplasm of the tumour cell population (Figure 3.8 below).

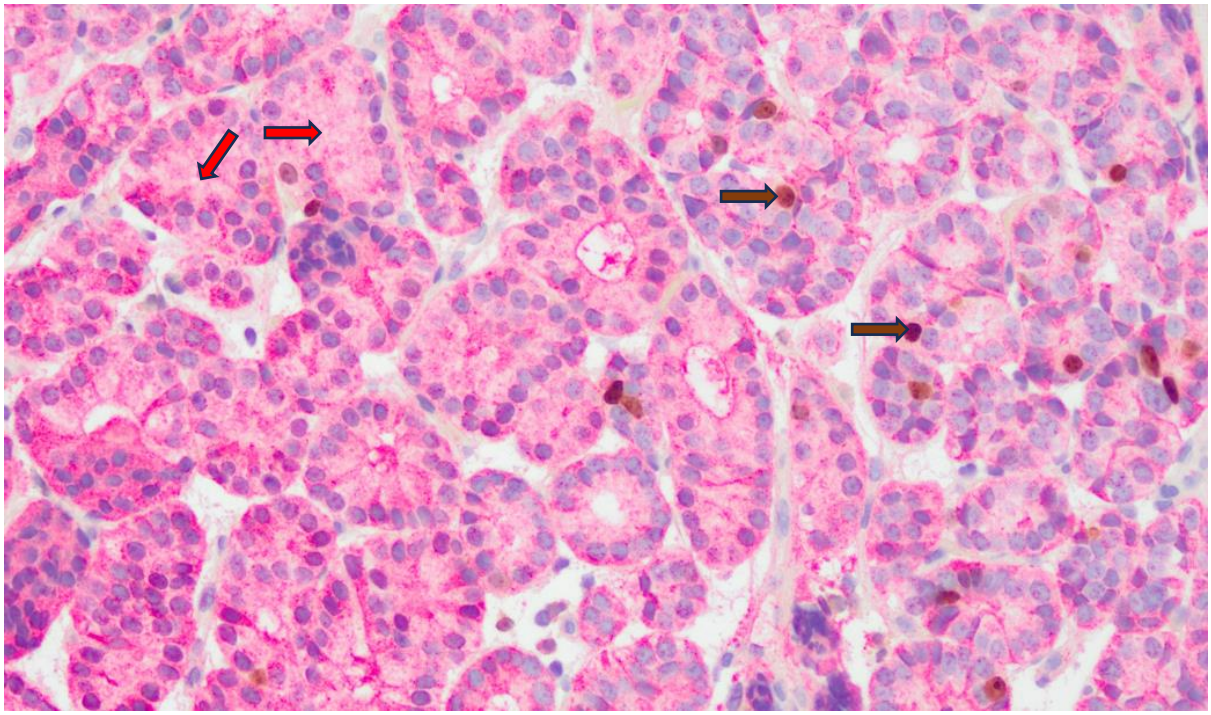

Figure 3. 8. Optimal Staining CgA-Ki67 DS method.

Magnification 200x. Chromogranin A appeared crimson red (red arrow and cytoplasmic stain) and the nuclear marker Ki67 appeared dark brown (brown arrow and nuclear stain).

The flow diagram below showcases the strategic plan undertook to optimise the Chromogranin A-Ki67 double staining method. As stated previously, the preliminary trials were used to define direction to take in terms of optimising the methods in trials and this was done by looking at the quality of stains yielded by the staining protocols used which were CgA 1:100/ Ki67 1:100 (ER1 20 min vs ER2 20 min) and CgA 1:100/ Ki67 1:50 (ER1 20 min vs ER2 20 min) (Figure

3.9 below). Showing in Table 3.11 and Figure 3.10 below, the intensities of the stain were too strong with background staining when the concentration of both markers were very high. The red chromogen in the cytoplasm of the tumour cells obscured any of the nuclear staining. Effectively Ki67 was only prominent outside of the tumour nests where there was no red chromogen present (Figure 3.10). These protocols were discontinued but set a starting point for the optimisation of the staining method. Antibody dilution 1:50 for the Ki67 marker was preferred.

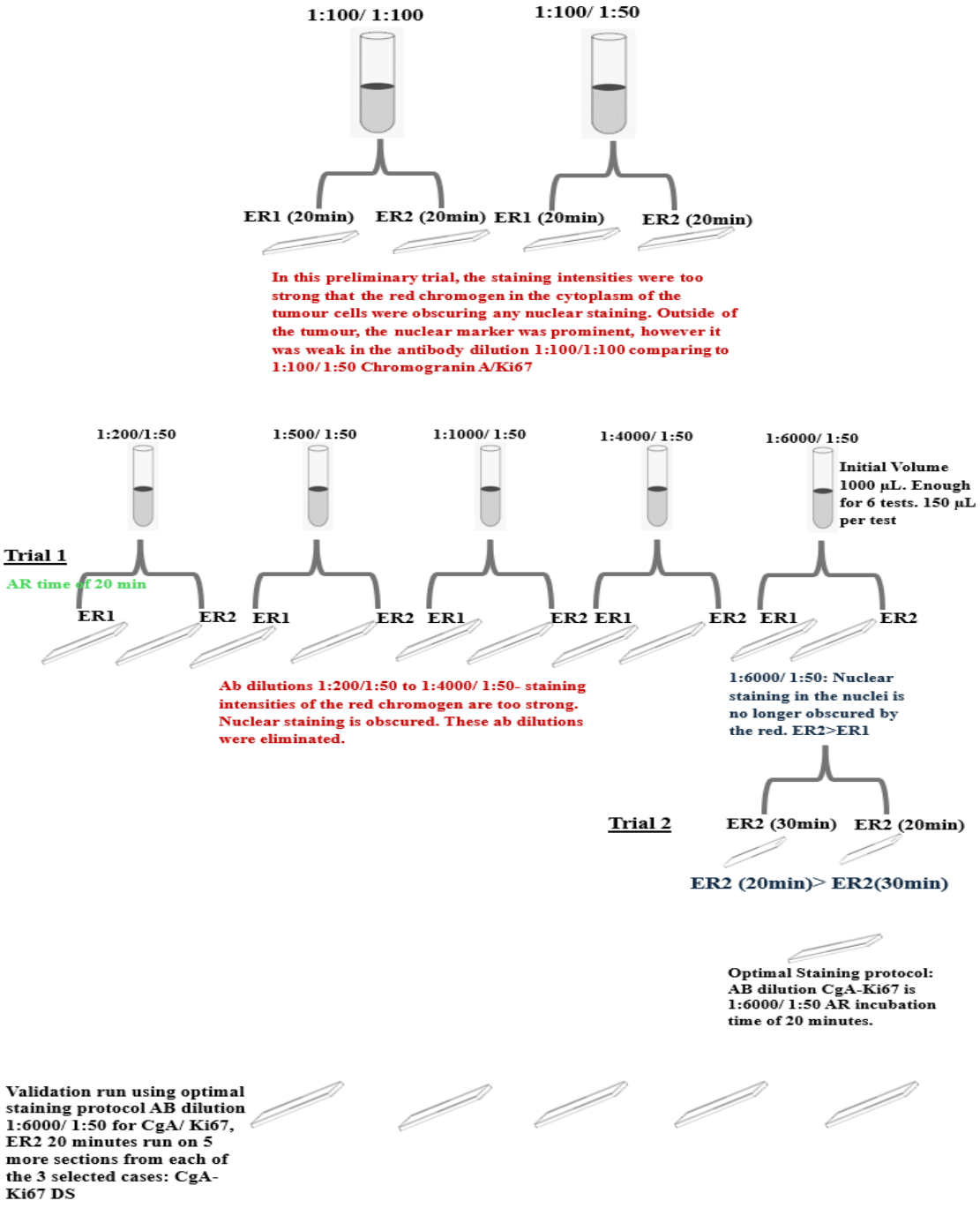

Figure 3. 9 Flow Diagram of the optimisation and validation of the CgA-Ki67 DS method.

Protocols of the preliminary trials were used to define direction to take in terms of optimising the CgA-Ki67 DS method. Protocols with stronger concentration of both antibodies were discontinued due to chromogen red obscuring the Ki67 nuclear markers.

Following Experimental Trial No.1 of the optimisation of the Chromogranin A-Ki67 double staining method, antibody dilutions (1:200/ 1:50, 1:500/ 1:50, 1:1000/ 1:50, 1:4000/ 1:50) were discontinued regardless of the antigen retrieval methods used. This was done due to the staining intensities of the chromogen red CgA still being overly strong and obscuring the nuclear Ki67 markers. From this trial, only the antibody dilution 1:6000/ 1:50 yielded staining that was not overly strong or red. With the focus now on the ab dilution CgA 1:6000 and Ki67 1:50 antigen retrieval method ER2 in Trial no.2, the intensity of the staining decreased when the antigen retrieval time increased from 20 to 30 minutes (Table 3.11 and Figure 3.10 below).

Table 3. 11. Result from the optimisation of the CgA-Ki67 DS method.

No. of Trial	Antibody dilution	Antigen Retrieval Method (ER1 or ER2 and time in minutes)	Sensitivity of Stain	Background stain
Trial No. 1	1:200/ 1:50	ER1(20)	Very strong staining esp. the red chromogranin A	High background stain
		ER2(20)		
	1:500/ 1:50	ER1(20)		
		ER2(20)		
	1:1000/ 1:50	ER1(20)	Very strong staining esp. the red chromogranin A	High background stain
		ER2(20)		
	1:4000/ 1:50	ER1(20)		
		ER2(20)		
	1:6000/ 1:50	ER1(20)	++ CgA + Ki67	Some
		ER2(20)	+++ CgA ++ Ki67	Low
Trial No. 2	1:6000/ 1:50	ER2(20)	+++ CgA ++ Ki67	Low
		ER2(30)	+ CgA + Ki67	Some

Key: ***Qualitative Score: (-) negative stain (+) weak positive stain (++) moderate positive stain (+++) strong positive stain.***

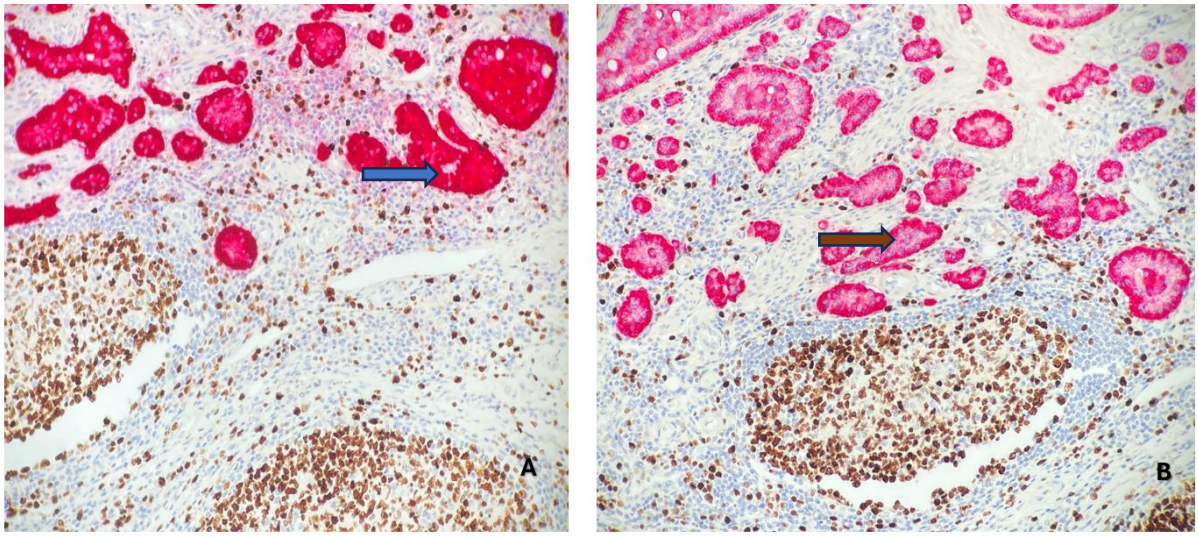

Figure 3. 10. Comparison of different antibody dilutions CgA 1:100/ Ki67 1:50 vs CgA 1:6000/ Ki67 1:50.

Magnification 100x (Image A): Antibody dilutions CgA 1:100/ Ki67 1:50. Showing by the blue arrow are nuclei obscured by the intense chromogen red CgA markers. (Image B): Antibody dilutions CgA 1:6000/ Ki67 1:50. Showing by the brown arrow are clear nuclei not being obscured or masked by the cytoplasmic CgA markers.

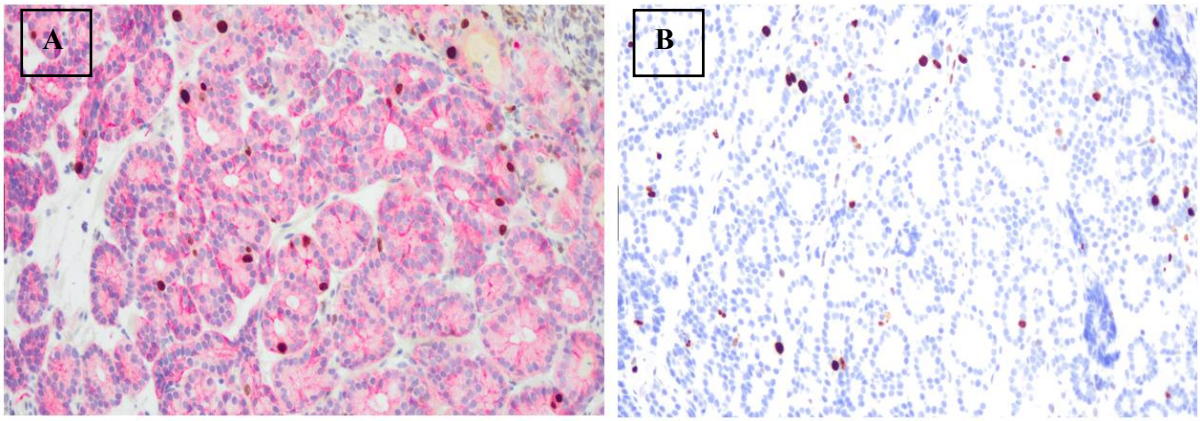

Figure 3. 11. Comparison of the Synaptophysin-Ki67 to the Ki67-only stain.

Magnification 200x. This gastric biopsy stained with the double staining method Syn-Ki67 (A) and on the right (B) is the same gastric biopsy stained with the gold standard Ki67 stain only. Both were stained with the optimal staining protocols for each staining method.

3.3.4 Validation Results

Table 3. 12. Results of the Validation Trials

Staining Method/ Validation No.	Study ID/Case ID/ Tissue type of control used	Validation1	Validation 2	Validation 3	Validation 4	Validation 5
Ki67-only stain	Tonsil	☑	☑	☑	☑	☑
Double stain Syn-Ki67	Case 1 19/P21340.1B	☑	☑	☑	☑	☑
	Case 10 23/P22529.1D	☑	☑	☑	☑	☑
	Case 5 22/P26103.1A	☑	☑	☑	☑	☑
Double stain CgA-Ki67	Case 1 19/P21340.1B	☑	☑	☑	☑	☑
	Case 10 23/P22529.1D	☑	☑	☑	☑	☑
	Case 5 22/P26103.1A	☑	☑	☑	☑	☑

All five of the validation slides for Ki67 passed the QC check. All showed optimal staining. This is a robust measure of repeatability 100 percent of the validation checks for the two double staining methods Synaptophysin-Ki67 and Chromogranin A-Ki67 were successful in showing optimal staining regardless of the types of specimens used, i.e. whether it is from a core biopsy or subsample from a tumour resection.

3.4 Discussion

The objective of this study was to optimize and validate staining protocols (Ki67-only stain, Synaptophysin-Ki67 double staining method and Chromogranin A-Ki67 double staining method) (Figure 3.10 & Figure 3.11) that will be used in later stages of this research. It was an important step in this study making sure that the proposed double staining methods can reproduce similar outputs to that generated but the gold standard Ki67-only stain and showed evidence of consistency and repeatability. The primary goal for optimising new antibodies in immunohistochemistry is to increase the specificity and improve sensitivity of the markers binding to discrete targeted antigens and minimize non-specific background signals (51). Sensitivity and specificity ideally work together with a single discrete marker, i.e. an antigen that is restricted and only present in the cell population of interest. The proliferation marker Ki67 is specific for an antigen but is not specific to a cell type. As a result, these experiments have the diagnostic goal of optimizing marker sensitivity and specificity for two antigens simultaneously, and to count only the cells that display both markers. Counting just the

proliferating cells, specific to the tumour population, rather than part of the host anti-tumour response, will boost the prognostic value of the KPI index. Once optimised and validated this improved tool should quantifiably benefit the diagnostic approach to GI NETs.

The optimisation of new antibodies is an essential step that must be taken prior to validation of selected staining parameters by performing IHC staining with the new primary antibody or antibodies (dual) on a set number of slides that include tissue sections with a wide range of reactivity (negative (-) to strong (+++)). Validation is mandatory to ensure reproducibility and consistency of results and once the method is exposed to the range of clinical specimens and preservation quality that is likely to be encountered. Both optimization and validation processes are significant undertakings where proposed new staining methods are introduced into the lab as new diagnostic tools. The optimisation of the three staining protocols in this study was achieved in multiple steps including, pre-analytical standardisation of tissue fixation, control selection, antigen retrieval and primary antibody dilution. Another consideration in optimisation is the cost of reagents that are involved, and this must be taken into account. The cost of an additional procedure to the lab must be weighed against the clinical benefits. In the context of this research the cost considerations come from undertaking the study in the first place and the future ongoing cost of a dual staining approach, if elected to supersede the existing method, previously considered a 'gold standard' i.e. the medium- and long-term benefits should be measurable.

The pre-analytical standardisation of tissue fixation in this study was achieved by the selection of control tissues and tissues of known cases from the laboratory archive. The following pre-analytical factors are known to influence quality of immunohistochemistry stains and were therefore important to be controlled or standardized; time delay between excision and fixation, type of fixative, concentration of fixative, pH and buffer of fixative, fixation time, reagents used during tissue processing cycles, temperature of the clearing reagent, the temperature of paraffin wax during embedding, the duration and temperature of slide drying (59). For these reasons, it was an advantage that all the tissues used in this study were processed internally from grossing to embedding. This means that since these tissues have been processed and passed through the MMH Histology Lab routine processes, it was expected that fixation and embedding requirements were met. However, it was still a significant task in our study to check that our selected cases met the criteria. The three cases selected for this part of the research were processed through the MMH lab workflow thus also provided reassurance of standard tissue fixation. The decision to only use three cases in this part of the research was primarily

due to the cost of the reagents. i.e. one antibody (Syn) cost NZ$953 and a Chromoplex detection kit of 50 tests cost NZ$756.14 dollars

Selection of appropriate positive and negative controls are essential in immunohistochemistry as validity of interpretations are based on it (52). It was also significant in our experiment to standardize control tissue at this stage for several reasons, and the main one is having the reassurance that tissue will not be damaged during antigen retrieval as a result of the tissue being under fixed. It was also important that we selected the right control to avoid wasted time, effort, and resources especially if targeted antigens of interest are not presence in the tissue controls used. Tonsil was selected as the positive control tissue for the Ki67 marker in both the 'Ki67-only' staining method and in the double staining methods (Syn-Ki67 and CgA-Ki67). Tonsil was the preferred tissue as a positive control because its surface epithelium has a clear Ki67 staining pattern, with high positive staining in the parabasal layer, low positive staining in the intermediate layer, and negative staining in the basal and superficial layers (7). The positive staining in these layers is due to the strong reaction of B cells in the dark zones of the germinal centres shown moderate to strong nuclear staining reaction (strong expression level of Ki67) indicating positive staining, while a weak to moderate staining are expressed by most B cells in the light zones (low expression level of Ki67) indicating negative staining. There is also no positive staining expressed by B cells in the mantle zone. All these observations and reasons why tonsil was the preferred positive control are consistent with the literature (11, 15, 33).Although tonsil could have been also utilized as a negative control in this study, we thought it would be better to choose a separate negative tissue control. For this reason, a normal liver tissue section was also placed alongside the tonsil section on the same slide to serve as a negative control and was successful (Figure 3.3 Panel B). This was done because it is known that fewer than 1% of the hepatocytes in the liver tissue section would stain positive for Ki67 and there was no cytoplasmic staining. In contrast, the double staining methods used a tonsil (Ki67) and a pancreas (Synaptophysin and Chromogranin A). Pancreas used in the double staining experiments showed strong red chromogen in the islets of Langerhans and in the peripheral nerve fibres (Figure 3.12).

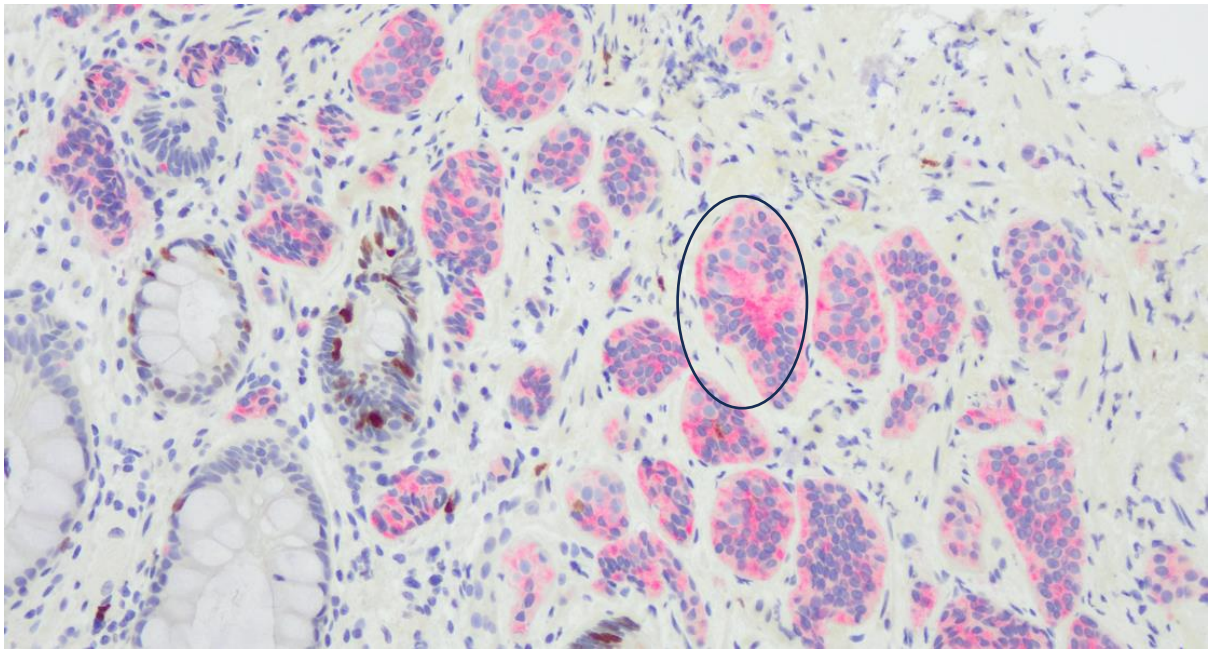

Figure 3. 12. Synaptophysin-Ki67 stain of a pancreas.

Magnification 400x. Shown above (inside the blue circle) are clusters of neuroendocrine cells stained with the red chromogens.

While two antigen retrieval methods are often used to retrieve single antigen (i.e. heat with buffer reagents or heat with enzymes), it can be also used to retrieve two antigens such as in the proposed double staining methods. However, there is the risk of affecting the integrity of the tissue. Because of this reason, our study settled on HIER (Heat-induced epitope retrieval) using Tris EDTA pH 9 buffer solution for 20 mins (Syn-Ki67 and CgA-Ki67staining method) and 30 min ('Ki67-only' staining) at 100 degrees Celsius as its method of choice. We also chose Heat-induced epitope retrieval (HIER) over the Protease-induced epitope (PIER) in this study for two main reasons. First, the method was successfully used in the study of Matsukuma et al (9) thus make sense we used similar approached so we can replicate similar outcome. Second, PIER is less frequently used as they are known to damage the morphology of the tissue and even the antigen itself. This is due primarily to the use of enzymes such as Proteinase K, Trypsin, and pepsin to degrade or destroyed the peptides masking the antigens which tend affect the tissue morphologies as well. PIER is also slightly longer than the HIER method thus unsuitable for diagnostic setting. The antigen retrieval method in this study involves two important parts (antigen retrieval solution ER and ER2 & antigen retrieval time) and were trialled independently from each other. The other factor that can influence antigen retrieval is time. Our study found that intensity of the staining improved as the antigen retrieval time increased. This indicated that allowing enough time for antigen retrieval to take place allows

more antigen to be unmasked and increase intensity of this stain. This outcome correlates with the literature. (57).

The concentrations of the antibodies (antibody dilutions) were undoubtedly the most significant factors influencing optimisation of each staining methods in this study impacted both the intensity and specificity of the stain. At high concentrations, the intensity of each staining methods increases and most often resulted in non-specific background staining. Non-specific background staining is known to arise because of excess primary antibodies, and this was seen in our study especially in the optimisation of the chromogranin A/Ki67 double staining method. The washing step employed after the incubation of the section with the primary antibody supposed to wash off excess unbound antibodies (Step 7 of Figure 3.3 and Step 9 of Figure 3.5). However, sometimes the washing step is not enough to wash off excess and unbound antibodies resulted in the need to reduce the concentration of the Chromogranin A as the red chromogen were masking the nuclear Ki67 antigens in the tumour nest (Figure 3.11 above). The problem was resolved by reducing the concentration of the primary antibodies i.e. increasing the antibody dilution of CgA to1:6000 in the CgA-Ki67 double staining method contrast, a very high concentration (1:50) of the Ki67 antibody was used in the cocktail. A completely different concentration to when Ki67 was optimised on its own. It is not understood why this has happened, but it could be due to several reasons including the use of the antibody cocktails (where the two antibodies where combine) and the antigen retrieval method used.

Other factors such as the antibody incubation time, incubation temperature, and antigen retrieval temperature, were kept constant throughout the experiment. These variables are in their default setting on the Leica BOND III platform as set by the manufacturer and are shown in Table 3.3 and Table 3.5 above. All of the staining protocols in the platform have the same settings for each of these factors. For instance, the antigen retrieval temperature is 100 degrees Celsius for every single protocols regardless. This is a possible limitation of this study that not all the factors that can influence an optimal staining of any antibodies/ or staining methods were being optimized. Following the optimisation step, each of the optimal staining protocols determined above were validated for reproducibility and consistency by further staining five different slides (Table 3.12 above). Although 100 percent of the validation slides were positive and passed the quality control checked for staining quality, this could be another possible limitation of this study. To successfully validated a new marker/ antibody/ IHC staining method, it is strongly recommended that laboratories should test or trialled protocols on a minimum of ten positive tissues and negative tissues (60). This could have been avoided in our

study if funds and reagents were not limited. Furthermore, the main source of limitation in this study was the cost of reagents as it impacted a lot of decision making and planning of this project. This was reflected in numerous decisions such as the used of only three cases for the optimisation experiment, five slides for validation experiments, and the choice to use antibody cocktails (simultaneous method) instead of applying the antibody sequentially in the optimisation of the double staining methods.

In future studies, optimisation attempts can omit repeating antigen retrieval solution ER1 (citrate buffer) since they have been shown not be an ideal antigen retrieval solution in all three of the optimisation experiments (Ki67-only stain, Syn-Ki67 DS method and CgA-Ki67 DS method). Doing this will allow researcher or the technician to concentrate instead on other variables related to time of antigen retrieval and antibody incubation time. Evidence for an inverse relationship between antibody concentration and incubation time in terms of sensitivity and specificity could be explored in a larger optimisation trial. Reducing the amount of antibody can potentially reduce background staining, making interpretation easier and extracting more diagnostic tests from the antibodies (i.e. more cost-effective diagnostic tests) although other factors such as antigen retrieval method selected can also plays a part. There would be no significant detectable impact on turnaround time based in existing workflows and staffing within anatomic pathology departments.

3.5 Conclusion

Optimisation processes involve more than just determining optimal antibody dilution and antigen retrieval methods, pre-analytical standardisation, control selection, antigen retrieval and primary antibody dilution. The process also takes into accounts cost of reagents and level of expertise of the technician and pathologists involved. Proper optimization is obviously essential for successful immunohistochemical (IHC) testing. An optimal IHC protocol can be characterised by its ability to consistently and unambiguously determine how the antigen is expressed in the tissue. In conclusion, we were able to successfully optimize all three of the staining methods Ki67-only stain, Syn-Ki67 and CgA-Ki67 which were achieved through a series of trial and experiments where the antibody concentrations and antigen retrieval methods were modified accordingly to the quality of the stains. As a result, the optimal staining protocols for the optimized staining methods were the following: **Ki67-only stain** *AB dilution 1:100 ER2:30 minutes*; **Synaptophysin-Ki67 stain** *AB dilution 1:50 for both antibodies ER2: 20 minutes*; **ChromograninA-Ki67 stain** *AB dilution CgA 1:6000 and Ki67 1:50 ER2 20 minutes*.

These optimal staining protocols were successfully validated by running them on a set of five slides to check for reproducibility and consistency of results. In conclude, 100 percent of the Ki67-only validation slides were validated successfully with the Ki67 expressed predominantly by the B cells in the dark zones of the tonsils. All fifteen of the validation slides for the two double staining methods Syn-Ki67 and CgA-Ki67 passed the validation trial. In these slides, the red chromogen of the cytoplasmic markers Synaptophysin and Chromogranin A did not mask the nuclear Ki67 staining in the tumour nest. This is significant finding progressing to the second part of this research where the performance of each staining methods in regard to Ki67 proliferative index quantification were evaluated.

4.0 Comparison of the Ki67-only staining method to the double staining methods.

4.1 Introduction

The idea of highlighting neuroendocrine tumour cells using two immunohistochemistry markers was initially conceptualised by Matsukuma and colleagues in their 2017 publication, in which they introduced the use of the Synaptophysin-Ki67 dual staining method for their assessment of KPI (9). More recently Ahn and co-authors have applied this double staining method focusing on a pancreatic subset of neuroendocrine tumours (39). Our study shared similar interest to that of the Matsukuma study as we were both focusing on the benefit of using double staining methods to quantify Ki67 proliferative index in gastrointestinal neuroendocrine tumours. This study is the first to report the use of the double Chromogranin A-Ki67 staining method to measure the KPI in the grading of well-differentiated gastrointestinal neuroendocrine tumours. It is also the first study to use concentrated antibodies in the optimisation of both Syn-Ki67 and CgA-Ki67 instead of ready to use antibodies from the manufacturer.

Matsukuma and colleagues found that the use of the double staining method in the assessment of the Ki67 proliferative index improved the concordance rate between observers, i.e. there was greater agreement over the Ki67 proliferative index in GI NETs (9). It was intuitive that highlighting tumour cells with markers binding to a neuroendocrine cell-specific antigen would makes tumour cells stand out from the rest of the cells. This would show the tumour population as distinct from surrounding tissue and infiltrating defensive or inflammatory cells. Once recognized by synaptophysin, Ki67 staining could be more confidently associated with a tumour cell or a cell adjacent to the tumour population. Of prognostic importance is the Ki67 proliferative index of the tumour cells, not a measurement that includes normal host defensive or stromal cells.

The use of dual (double) or multiple IHC markers in diagnostic pathology is growing, with many now being utilized in various applications. This is primarily due to greater ease of interpretation from less time-consuming manual counts by pathologists together with, higher reproducibility and accuracy. The use of dual IHC staining has been well established in other areas, for instance the use of the p16-Ki67 dual IHC staining method in the screening and triaging of cervical cancer and precancerous lesions (61). Ki67 and p16 are used in this

application in a similar fashion to how Ki67 is being used with GI NETs study, a proliferative marker using alongside a cytoplasmic marker. Dual Stains cyclin D1/ CD79 and PAX5/CD5 are known to be useful in the detection of mantle cell lymphoma (MCL) in bone marrow (BM) in which recognition of neoplastic B cells can be a challenge due to insufficient or low level expression of B cells in the disease situation (62). All these multiple IHC staining protocols are possible due to antigens being from different parts of the cells without overlapping each other. i.e. PAX5 is a nuclear antigen and CD5 are found in the cytoplasm.

These double staining methods share some common themes. The main one is high sensitivity for identifying tumour cells in their individual applications making it easier and less time consuming for the user or pathologists to look at and identify tumour cells. Dual stain methods commonly target markers in different cell compartments as a way to keep interpretation straight forward. Last but not the least, dual stain methods are commonly developed to overcome diagnostic challenges with existing methods and need to be compared and validated against a method that is already in use and familiar to pathologists. This provides labs with a different problem. How to show the utility of a new diagnostic method when it is being compared with a 'gold standard' method that it might well supersede. This is a good problem to have but there is a relative lack of standardised approaches with which to approach this challenge.

In this part of the research, the objectives were to validate the reproducibility of the proposed double staining methods against confirmed positive well-differentiated gastrointestinal neuroendocrine tumours (WDGI NETs) cases and to evaluate the impact of specimen type (biopsy vs resection), location or origins of specimens in the GIT tract, and staining methods (double stains vs Ki67-only) on the assessment of Ki67 Proliferative Index. Furthermore, it will determine whether the proposed double staining methods provide a better outcome than the current gold standard Ki67-only stain. This is an approach that was successfully demonstrated in the study of Obiorah and coauthors demonstrating CyclinD1/CD79a and PAX5/CD5 dual IHC stains to not only comparable to the gold standard method Flow Cytometry but superior to single IHC staining methods for the detection of Mantle Cell Lymphoma in bone marrow (62).

4.2 Methods and Materials

4.2.1 Selection of Cases

A total of 20 confirmed positive well-differentiated gastrointestinal neuroendocrine tumour (GI NETs) cases were selected for this study from a pool of 134 confirmed gastrointestinal neuroendocrine cases diagnosed at the Middlemore Hospital Histology (MMH) Lab, Auckland, New Zealand between the year 2015 to 2024. This study only focused on well-differentiated gastrointestinal neuroendocrine tumours because poorly differentiated tumours could easily have less consistent expression of synaptophysin and chromogranin A at the same time as having an aggressive neoplastic phenotype and high Ki67. It was also relatively pointless to perform a double stain to measure Ki67 index if the routine H&E stain confirmed a poorly differentiated gastrointestinal neuroendocrine tumour and direction for treatment. The MMH Laboratory Delphic AP archive was accessed and searched for positive eligible cases using the search phrases "neuroendocrine tumour cases", "gastrointestinal neuroendocrine tumour", "well differentiated gastrointestinal neuroendocrine cases". Cases were selected for this study based on the following inclusion criteria; positive well-differentiated GI NETs, availabilities of blocks and slides, must either be a biopsy or resection specimen, sufficient diagnostic material available (enough tissues left in the blocks), adequate fixation. Gastrointestinal biopsy specimens received in our laboratory arrive as either a core needle biopsy or endoscope biopsy. Cell blocks were excluded from this study as they do not contain sufficient diagnostic material. Cell blocks can be prepared from most cytology specimens such as fine needle aspirations, body cavity fluids, washings, brushings and gynecologic and nongynecologic liquid. They are usually the size of a button and normally after the initial H&E staining and traditional additional tests such as special stains/immunohistochemistry/molecular tests, not a lot of the cell block is left for test development. In diagnostic histopathology, it is common to have more than one FFPE tissues and several paraffin blocks per cases. To avoid wasting research time and money, the diagnostic report for each of the 20 cases was used as a guide to identify the best block (sufficient diagnostic material and containing the tumour) from each case for our study. Specimens were derived from various sites (2 liver, 2 duodenum, 1 ileum, 6 appendix, 1 pancreas, 5 stomach, 3 rectum) as it was important to evaluate the reproducibility of the proposed double staining methods on different encountered in lab submissions.

4.2.2 Pre-staining processes

4µm tissue sections were cut from each of the twenty formalin-fixed paraffin embedded (FFPE) tissues and transferred onto microscopic glass slides (3 slides corresponding to each of the staining methods; Ki67-only stain, Syn-Ki67 double stain or CgA-Ki67 double stain). A control section for each of the three staining methods (Ki67-only, Syn-Ki67 and CgA-Ki67 double staining methods) cut at 4 microns was also added to each of these slides. The sections were then baked in a 60℃ oven for 30 minutes to heat fix them onto the glass slides before staining. An H&E-stained section was examined to confirm the selection criteria effectively provided well differentiated GI NETs.

4.2.3 Immunohistochemistry Staining Protocol

Like the optimisation and validation experiments in the previous chapter, all the IHC staining trials in this part of the study were done in batches in the Leica BOND III. It also used the same protocol (Figure 4.1 & 4.2) trialled in the optimisation and validation experiment in the previous chapter but with the determined optimal staining protocol.

4.2.3.1 Gold Standard (Ki67-only stain)

Step	Reagent	Time (min)	Temperature (°C)
1	Bond Dewax Solution x 3 changes	30	72
2	Alcohol x 3 changes	0	RT
3	Bond wash solution x 3 changes	0	RT
4	Bond ER Solution **ER2**	**30**	100
5	Bond wash solution x 5 changes	3	RT
6	Primary antibody Ki67 Various concentration or antibody dilution: **1:100**	15	RT
7	Bond wash solution x 3 changes	0	RT
8	Post-primary	8	RT
9	Bond wash solution x 3 changes	6	RT
10	Polymer	8	RT
11	Bond wash solution x 2 changes	4	RT
12	DI water wash	0	RT
13	Peroxide block	5	RT
14	Bond wash solution x 3 changes	0	RT
15	Mixed DAB refines detection kit	10	RT
16	DI water wash x 3	0	RT
17	Haematoxylin	7	RT
18	DI water wash	0	RT
19	Bond wash solution x 1 change	0	RT
20	DI water wash	0	RT

Figure 4. 1. IHC Staining Protocol for the Ki67-only staining method.

Optimal Staining Protocol*: AB dilution 1:100 ER2 at 30 minutes. KEY: RT- Room temperature. ER2-Epitope retrieval solution 2.*

4.2.3.2 Double Stains IHC protocol (Syn-Ki67 and ChromA-Ki67)

Step	Reagent	Time (min)	Temperature (°C)
1	Bond Dewax Solution x 3 changes	30	72
2	Alcohol x 3 changes	0	RT
3	Bond wash solution x 3 changes	0	RT
4	Bond ER Solution **ER2**	**20**	100
5	Bond wash solution x 5 changes	3	RT
6	Peroxide Block	5	RT
7	Bond wash solution x 3 changes	0	RT
8A **8B**	Cocktail A (Syn-Ki67 ab dilution **Syn 1:50/ Ki67 1:50**) Cocktail B (CgA-Ki67 ab dilution **CgA 1:6000/ Ki67 1:50**)	15	RT
9	Bond wash solution x 3 changes	0	RT
10	Polymer mHRP	8	RT
11	Bond wash solution x 3 changes	0	RT
12	Polymer rAP	20	RT
13	Bond wash solution x 5 changes	5	RT
14	DI water	0	RT
15	Mixed DAB refine detection kit	10	RT
16	DI water x 3 changes	0	RT
17	Red chromogen added (Mixed Red kit)	15	RT
18	DI water x 3 changes	0	RT
19	Haematoxylin	5	RT
20	DI water	0	RT
21	Bond wash solution x 1 wash	0	RT
22	DI water	0	RT

Figure 4. 2. IHC Staining Protocol for the two proposed double stains.

Synaptophysin-Ki67 and ChromograninA-Ki67. ***Optimal Staining Protocol****: Synaptophysin-Ki67 (AB Dilution for both antibodies 1:50 ER2 for 20 minutes) ChromograninA-Ki67 (AB Dilution CgA 1:6000; Ki67 1:50 ER2 for 20 minutes). KEY: RT- Room temperature. ER2- Epitope retrieval solution 2.*

4.2.4 Post-staining processes

Following the final washing step in DI water (Figure 4.1- Step 20 and Figure 4.2- Step 22), the slides were unloaded from the Bond III machine, dehydrated in two changes of alcohol to remove excess water from the sections and then cleared in two changes of xylene before cover slipping in the Leica CV5030 Fully Automated Cover Slipper. Each of the 3 replicates was checked for quality of staining both on control and test material, and that each slide had the correct control (tonsil for the Ki67 stain only and tonsil/pancreas for the double stains Syn-KI67 and CgA-Ki67). The cases were randomly allocated to the reading pathologists for the assessment of the Ki67 proliferative index (KPI).

4.2.5 Randomisation of the study cases

Each of the 20 cases was given a random study number (Study ID) of 1 to 20 and were then randomly allocated to the reading pathologists for blind scoring (Ki67 Proliferative Index). The lab numbers and patients IDs of each of the 20 cases were always kept unknown from the readers. These details can be found in the table below (Table 4.1).

Table 4. 1. Study ID and demographics of the 20 study cases.

Study ID	Lab No.	Block No.	Site of Lesion	Sex	Ethnicity	Age (Years)
1	19/P21340	1B	Liver	F	European	63
2	19/P21425	1A	Liver	M	Maori	64
3	21/P01991	1B	Duodenum	M	Indian	61
4	21/P03446	1A	Appendix	F	Pacific	30
5	22/P26103	1A	Duodenum	F	Asian	49
6	23/P14371	1A	Appendix	M	European	54
7	23/P18690	1A	Rectum	F	Maori	63
8	23/P20462	1C	Ileum	F	European	32
9	23/P21472	3A	Rectum	F	European	50
10	23/P22529	1D	Appendix	M	European	24
11	24/P01331	1E	Pancreas	F	Asian	52
12	24/P02004	2A	Stomach	F	European	69
13	22/P02185	5A	Rectum	F	European	73
14	21/P24503	1A	Appendix	F	Arab	32
15	22/P07160	1A	Stomach	M	European	72
16	22/P10118	1L	Stomach	M	European	70
17	22/P09909	1D	Stomach	M	European	72
18	22/P10120	1B	Appendix	F	European	21
19	22/P07280	1A	Stomach	M	European	58
20	22/P05609	3A	Appendix	F	European	36

4.2.6 Manual Counting of proliferative cells on captured/printed image.

Counting the proliferating cells for both Ki67-only stain and the two double staining methods (Syn-Ki67 and CgA-Ki67) was conducted using the method shown below. All counts were performed using Olympus BX53 microscopes. Each reading pathologists chose the magnification at which counting was conducted however they were instructed to blind score the allocated cases following the procedure below. They were also asked to comment on the quality of the stains, any difficult cases they came across and to provide as much feedback as practical.

1. Start Stopwatch.
2. Select “hot spot”/ a region of interest with the highest number of Ki67 stained nuclei.
3. Count number of positive cells.
4. Count number of negative cells.
5. Add positive and negative cells to reach the denominator.
6. Calculate the Ki67 Proliferative Index: positive cells/ total cells counted and express as a percentage by multiplying the value by 100 (%)
7. Save image.
8. Stop stopwatch.

4.2.7 Statistical Analysis

Statistical analysis of the data collected in this research was performed using the IBM SPSS version 29.0.2.0 (20) software and Microsoft excel software. The correlation between the gold standard method (Ki67-only) and each of the proposed double staining methods were calculated using the IBM SPSS software to validate the reproducibility of each of the proposed Syn-Ki67 and CgA-Ki67 staining methods. The correlation between the two proposed staining methods themselves was also calculated to validate reproducibility of the newly CgA-Ki67 method and its reliability. The intraclass correlation coefficient (ICC) with 95% confidence intervals were calculated to assess whether the inter-rater agreement on Ki67 proliferative index (KPI) for each of the staining methods (Ki67-only stain vs Syn-Ki67 double stain; Ki67-only stain vs CgA-Ki67 double stain; and Syn-Ki67 vs CgA-Ki67) were significant or not. In addition, the Fleiss kappa κ was calculated to determine grade concordance between the three methods. We then calculated the differences in the mean KPI performances of each method from the overall mean KPI (all three methods) and compared using the Microsoft excel software. These were performed taking into regards the types of specimens and levels in the GIT system that the tumour came from. The efficiency of each method in conducting a KPI assessment was also compared to determine whether the proposed staining methods are better than the gold standard by comparing the mean difference in time taken by each of the three staining methods to complete an assessment of the Ki67 proliferative index. A paired t test was then used to compare the differences between the means of time taken (in minutes) to complete a Ki67 proliferative index assessment by each of the three staining methods (Ki67-only stain, Syn-Ki67 method, and CgA-Ki67 method) and whether the difference was statistically significant or not. The Kolmogrov-Smirnof test was also used to test the data for normal distribution. p-values were also calculated to determine whether relationships between

variables are statistically significant or not. This means that any p-value less than 0.05 is considered statistically significant and that the relationship between the two variables has not occurred by chance alone.

4.3 Results

All selected gastrointestinal neuroendocrine tumour cases were reviewed as meeting the criteria for well-differentiated and the block and section chosen confirmed to have adequate tumour material for further testing and comparison.

Table 4. 2. Demography of study participants.

Study ID	Site of Lesion	Sex	Ethnicity	Age (Years)
1	Liver	F	European	63
2	Liver	M	Maori	64
3	Duodenum	M	Asian	61
4	Appendix	F	Pacific	30
5	Duodenum	F	Asian	49
6	Appendix	M	European	54
7	Rectum	F	Maori	63
8	Ileum	F	European	32
9	Rectum	F	European	50
10	Appendix	M	European	24
11	Pancreas	F	Asian	52
12	Stomach	F	European	69
13	Rectum	F	European	73
14	Appendix	F	Arab	32
15	Stomach	M	European	72
16	Stomach	M	European	70
17	Stomach	M	European	72
18	Appendix	F	European	21
19	Stomach	M	European	58
20	Appendix	F	European	36

A list of the patient demographics associated with the selected cases, and the location of the tumour is provided (Table 4.2). Eight (40%) were cases from male patients and 12 (60%) from female patients. Thirteen (65%) of these cases were European, 2 Māori (10%), 1 Pacific Islander (5%), 3 Asian (15%), and 1 other ethnic group (5%). Three (15%) cases were from patients either younger than 30 years of age or older than 70. Six (30%) were from patients (30-50 years old); and eight (40%) were from patients between 51 to 70 years of age. There were equal numbers of cases from both the upper (10 cases) and lower gastrointestinal tract (10 cases) with the highest number of cases by location coming from the appendix (30%) and the stomach (25%).

4.3.1 Gold Standard

The average number of tumour cell nuclei counted per tumour for the Ki67-only stain was 798 (range 502 to 1285). The mean Ki67 proliferative index (KPI) for all 20 tumours was 1.3 percent with 18 out of 20 cases (90%) in the 0.2-1.9% range, and one case each (5%) of the 20 within the 2.0-3.9% and 4.0-5.9% range respectively. The KPI range when using the gold standard method was from 0.2 to 5.2%. Nineteen cases (95%) were grade 1 and one case (5%) was grade 2. No cases were assigned to the grade 3 category.

Table 4. 3. Summary of the tumour size and KPI for each of the staining methods (Ki67-only, Syn-Ki67 and CgA-Ki67).

Study Number	Tumour size (mm)	KPI on Ki67 alone	KPI on (Syn-Ki67 DS)	KPI on (CgA-Ki67 DS)
1	1.2	1.7	2.9	2.3
2	2	1.3	1.9	2
3	5	0.6	1	1.1
4	1.7	2.8	0.6	1.0
5	4	0.8	0.6	0.7
6	20	1.0	0.7	0.8
7	9	1.1	2.2	2
8	10	1.4	2.6	2.1
9	2	0.6	1.3	1.5
10	20	1.7	0.2	0.3
11	16	1.7	1.2	1.2
12	2.7	0.7	2.5	2.3
13	2.6	0.4	0.7	-
14	15	1.2	0.3	0.2
15	20	0.5	1.4	1.9
16	21	1.2	0.5	0.4
17	20	0.2	1.4	1.6
18	3	0.9	0	0.2
19	6	5.2	5.9	5.7
20	23	1.7	0.6	0.4

Note: Missing result Study number 13 KPI for CgA-Ki67 was due to a machine technical error during the run.

Data Distribution

Because one study number, case 19, was grade 2 and had a higher KPI% than the tumours in the grade 1 category, it was important to test the data for a normal distribution, given that subsequent statistical testing required the assumption of normality. The Kolmogrov-Smirnof test for normality returned p-values of 0.0977, 0.3102, and 0.4168 for ‘Ki67-only’ stain, Syn-Ki67 stain, and CgA-Ki67 dual stains respectively. For all analyses requiring a 3 way comparison, study number 13 was omitted in order to balance the data.

4.3.2 Synaptophysin-Ki67 and ChromograninA-Ki67 Double Stain

The average number of tumour cell nuclei counted per tumour for the double stains was 743 (Synaptophysin-Ki67 double stain) and 700 (ChromograninA-Ki67 double stain) respectively. The mean Ki67 proliferative index for all 20 cases in each of the double stains was 1.4% (0 to 4.1) for the Syn-Ki67 and 1.5% (0.2 to 5.7) for the CgA-Ki67 staining methods. All 20 tumour cases were diffusely positive for both Synaptophysin and Chromogranin A. Proliferating cells from within the tumour population of each of the 20 tumour cases strongly demonstrated internal positive controls for Ki67 (Figure 4.3, Panel A). The intensity of each NET cell marker varied from case to case and ranged from moderate to strong depending on factors such as specimen types (i.e. biopsy vs resection) and tumour size. Regardless of the variation in staining intensity, the borders of the tumour in relation to the background stroma were sharp providing contrast and a clear distinction between the tumour and non-tumour area of the tissue (Figure 4.3, Panel B).

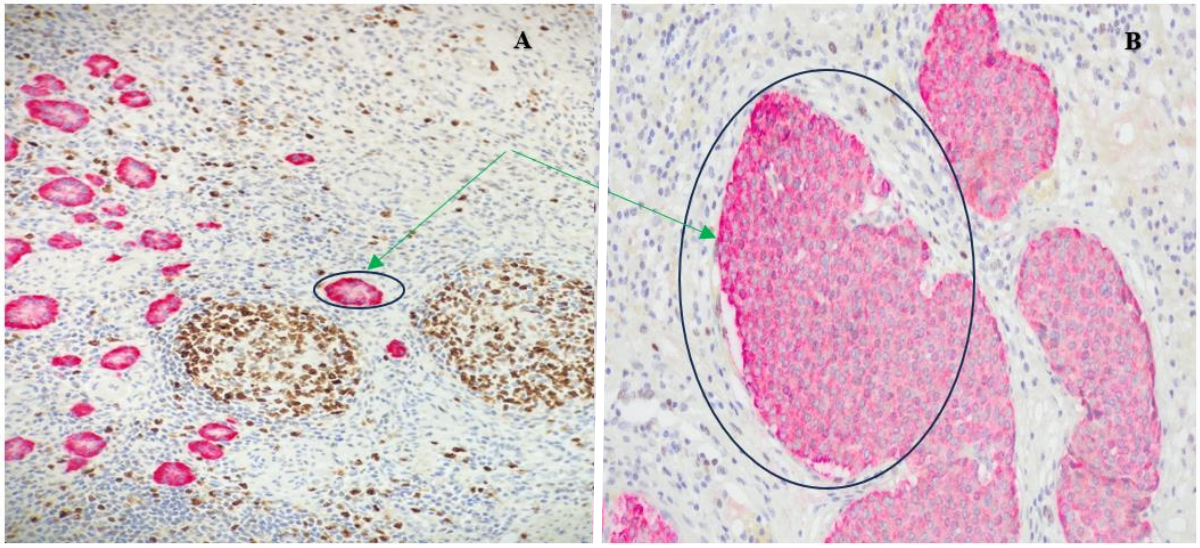

Figure 4. 3. Red chromogen presenting a clear distinction between the tumour and non-tumour area of the tissues.

PANEL A: Magnification 100x. Study ID 7 Case No. 23/P18690 (, Chromogranin A-Ki67). Lymphoid cell clusters adjacent to tumour islands are an internal control for Ki67.

PANEL B: Magnification 200x. - Study ID 6 Case No. 23/P14371 (, Synaptophysin-Ki67) illustrates the border of the tumour as clearly defined and separated from other healthy cells (Oval and arrows).

4.3.3 Validation of the double staining methods against the Gold Standard for reproducibility.

Table 4. 4. Intraclass correlations (ICC) in KPI Assessment between the gold standard 'Ki67-only' stain and the Proposed Staining Methods (Syn-Ki67 & CgA-Ki67).

Staining Methods	Intraclass correlation (95% confidence interval)	p-value
Ki67-only stain and the Synaptophysin-Ki67 DS method	0.633 (0.25-0.84)	0.003
Ki67-only stain and the ChromograninA-Ki67 DS method	0.627 (0.23-0.84)	0.004
Synptophysin-Ki67 and the ChromograninA-Ki67 DS method	0.982 (0.23-0.992)	<0.001

Intraclass correlation in the Ki67 Proliferative Index (%) of 20 well-differentiated gastrointestinal neuroendocrine tumours assessed by each of the three staining methods Ki67-only stain, Syn-Ki67 double stain and CgA-Ki67 double stain. 95% confidence intervals are in brackets, and both p-values are less than 0.05 showing that the correlation between the pairs are statistically significant.

Our data (Table 4.4 above and 4.5 below) presented strong correlations between the gold standard Ki67-only stain and the two double staining methods. The correlation coefficient for Synaptophysin-Ki67 with Ki67-only was 0.633 and for ChromograninA-Ki67 with Ki67-only was 0.627. The ICC between the two proposed staining methods Syn-Ki67 and CgA-Ki67 when compared was 0.982, presented an even stronger correlation than it was with the gold standard. All the intercorrelation measured in this experiment are statistically significant since p values in all comparisons are less than 0.005 (Table 4.4) and the 95 percent confidence intervals in all three comparisons made above did not include 0.

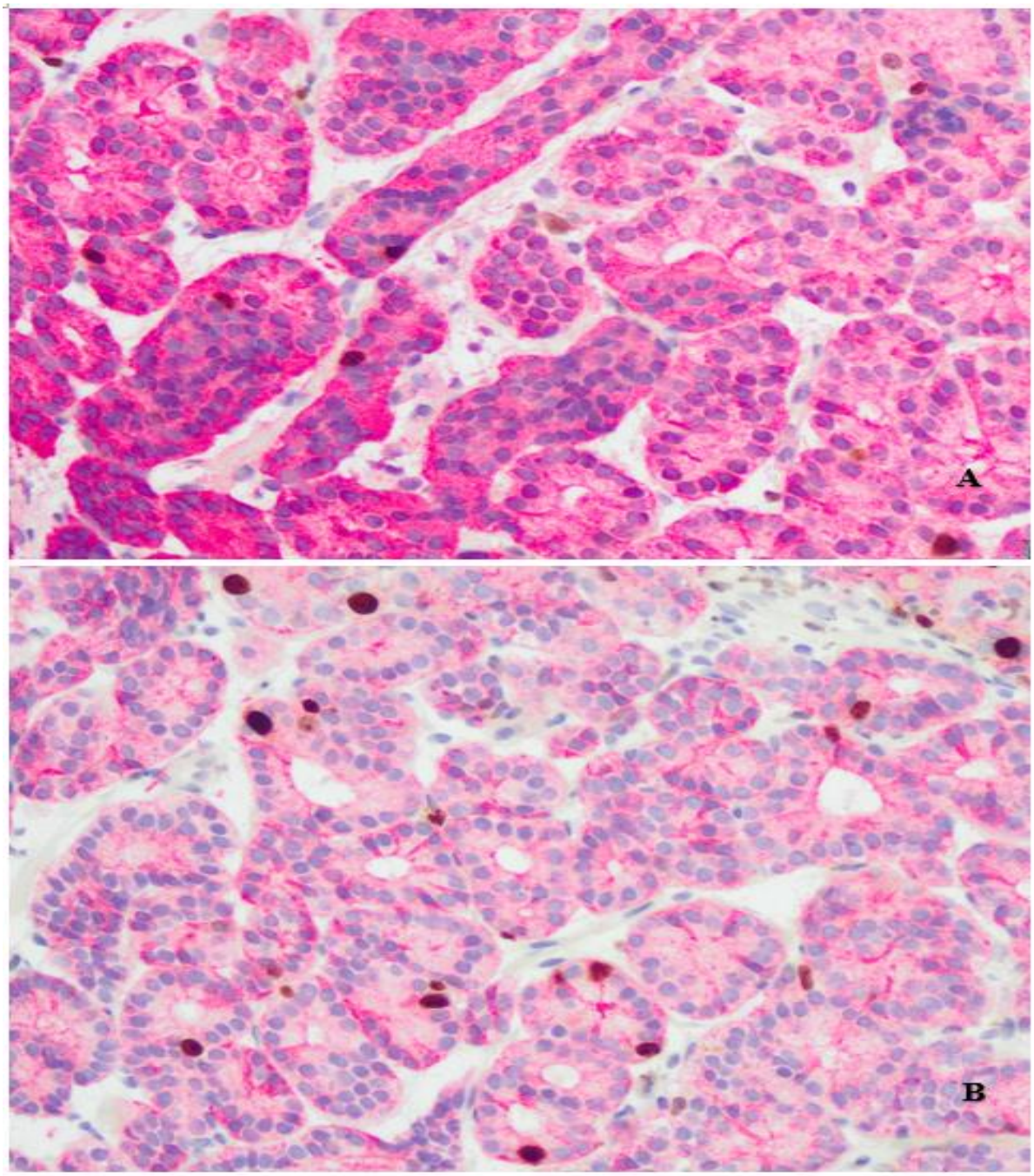

Figure 4. 4. Double staining methods (Syn-Ki67 vs CgA-Ki67).

Magnification 400x. ***Panel A****- CgA-Ki67 staining Case study no.15* ***Panel B****- Syn-Ki67 staining Case study no.15. Brown dots on both images- positive Ki67 stained nuclei of tumour cells.*

Table 4. 5. Multirater κ values for grade of 20 well-differentiated gastrointestinal neuroendocrine tumours using the gold standard Ki67-only and the two double staining methods (Syn-KI67 and CgA-Ki67).

Grading Category	κ-value (95% confidence interval)	P-value
Grade 1	1.0 (0.75 – 1.25)	<0.001
Grade 2	1.0 (0.75 – 1.25)	<0.001

None of the cases were in the Grade 3 category.

In the comparison of the tumour grades in Table 4.5, all three of the staining methods (Ki67-only, Syn-Ki67 and CgA-Ki67) strongly agreed that 95 percent of the 20 tumour cases were in the Grade 1 category (κ value= 1, p-value= <0.001, strong agreement) and only 5 percent in the Grade 2 category (κ value= 1, p-value= <0.001, strong agreement). Please note that this rating was not by the observer (reading pathologists) but the staining methods themselves.

4.3.4 Performance evaluation of the three staining methods.

Table 4. 6. Accuracy and precision of each of the three staining methods in measuring KPI% of well differentiated GI NETs cases.

Average KPI% of all 20 GIT NETs cases by all three staining methods and average KPI% for all three methods

No. of cases	Mean KPI% (all methods)	Mean KPI% Ki67-only stain	Mean KPI% Syn-Ki67 DS method	Mean KPI% CgA-Ki67 DS method
19	1.44	1.38	1.46	1.49

Mean Deviation of each KPI% assessment from the Mean KPI% assessment (all methods)

No. of cases	Mean KPI% (all methods) minus Mean KPI% Ki67-only stain	Mean KPI% (all methods) minus Mean KPI% Syn-Ki67 DS method	Mean KPI% (all methods) minus Mean KPI% CgA-Ki67 DS method
19	0.62	0.34	0.28

Pairwise t-test comparison of deviation between KPI% and each KPI% method

Compared variables	**p-value**
Ki67-only stain vs Syn-Ki67 DS method	< 0.0001
Ki67-only stain vs CgA-Ki67 DS method	<0.0001
Syn-Ki67 DS vs CgA-Ki67 DS	0.406

Because each of the Ki67 determination methods provided agreement on the grade of the test WDGI NETs cases they could be treated as replicates (although some assumptions regarding experimental replication were not able to be fulfilled) and that the mean of the determinations would be a better estimate of the true KPI% than any KPI% determination alone. We tested this hypothesis by comparing the difference between of the methods and the calculated mean KPI%. The Ki67-only method difference from the mean value (0.62) was notably greater than the Syn-Ki67 method difference (0.34) and the CgA-Ki67 difference (0.28) each a high degree of statistical difference ($p<0.0001$). This indicated that the Ki67-only method (gold standard) provided a more variable assessment of the true KPI% and/or that the tests were not independent replicates/ estimates. The two dual stain difference estimates, Syn-Ki67 (0.34) and CgA-Ki67 (0.28), were not statistically different from one another ($p= 0.406$).

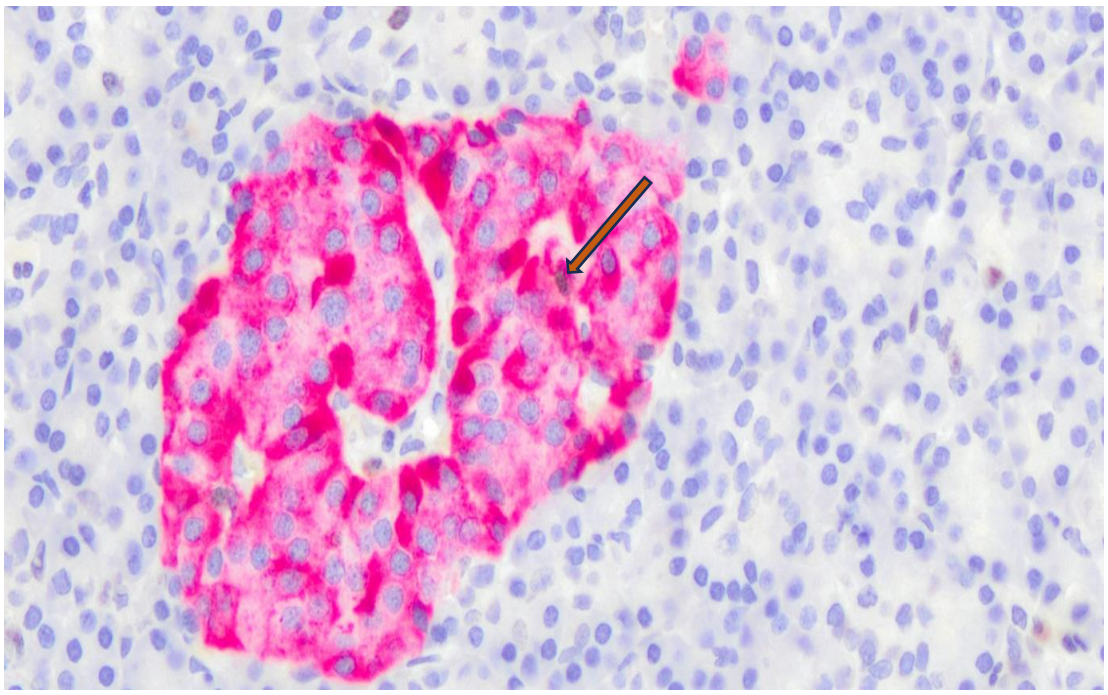

Figure 4. 5. Study Number 6 Case No. 23P14371.1A.

Magnification 400x. Appendix. In red is the Chromogranin A protein in the cytoplasm of neuroendocrine cells of both normal and neoplastic tissue. Stained in moderate to strong granular cytoplasmic staining of the neuroendocrine cells within the epithelial surface of this appendix. Brown arrow showing a DAB-stained nucleus elongated in shape almost hidden in the cluster.

Table 4. 7. Analysis time taken in minutes to perform KPI assessment in each of the methods.

	Staining Methods	Mean (mins)	Mean Difference	Standard error	p-value
Pair 1	'Ki67-only' stain	24.7	13.23	0.569	< 0.001
	Syn-Ki67	11.4			
Pair 2	'Ki67-only' stain	24.7	13.10	0.604	< 0.001
	CgA-Ki67	11.6			

Estimate is the mean of the difference of time (minutes) taken to complete a Ki67 proliferative index assessment on a log scale between the gold standard method and each of the proposed double staining methods (Syn-Ki67 and CgA-Ki67.

Shown in Table 4.7 above is the mean differences in time taken to complete a Ki67 proliferative index assessment between the gold standard method Ki67-only and each of the two proposed double staining methods taking into account the performance of the individual reading pathologists. In each of the comparisons (Pair 1 and 2), the double staining methods (Syn-Ki67 and CgA-Ki67) took a shorter time to complete the KPI assessment than the Ki67-only staining method. The paired t tests shows that differences between the double staining methods and the gold standard are significant. Each of the mean differences above has a p value less than 0.001 and a 95 percent confidence interval of 12.0 to 14.4 minutes for the Ki67-only stain time (min) vs Syn-Ki67 time (min) and 11.8 to 14.4 for the Ki67-only stain time (min) vs CgA-Ki67 DS time (min). These 95 percent confidence intervals excluded zero thus indicated mean differences that are statistically significant. These differences are also presented in the Panel of images in Figure 4.6 below where Panel B and C show DAB-stained nuclei independent of tumour cell clusters in contrast to Panel A. Without the dual stain, pathologists must decide the boundaries between tumour clusters and non-tumour hosts cells.

4.3.5 Factors affecting performance of the KPI determination methods.

Table 4. 8. Analysis of KPI by specimen types: Biopsy versus resection.

Specimen Type	Mean KPI% (all staining methods)	Mean KPI% Ki67-only stain	Mean KPI% Syn-Ki67 DS	Mean KPI% CgA-Ki67 DS
Biopsy (11 or 10)	1.696	0.53	0.30	0.29
Resection (9)	0.996	0.66	0.38	0.28

Pairwise t-test comparison of deviation between mean KPI% and each KPI% method (n=10/9)

Compared variables	Specimen Types	
	Biopsy	**Resection**
Ki67-only vs CgA-Ki67	p = 0.003	p = 0.001
Ki67-only vs Syn-Ki67	p = 0.001	p = 0.001
Syn-Ki67 vs CgA-Ki67	p = 0.840	p = 0.237

Biopsy specimens (n=11) returned a higher mean KPI % than resection specimen (n=9) as shown in Table 4.8. An unpaired 2-tailed students T-test indicated that this difference was not significant ($p = 0.177$). Only 10 biopsy specimens were included in pairwise comparisons. In the analysis of the KPI performances of the three staining methods in relation to specimen type subsets the mean differences were higher for the gold standard method (biopsy 0.53, resection 0.66), regardless of the specimen types, in comparison to the dual staining methods Syn-Ki67 (biopsy 0.30, resection 0.38) and CgA-Ki67 (biopsy 0.29, resection 0.28). Similar to results from the full dataset, the Syn-Ki67 and CgA-Ki67 DS methods were significantly less variable than the Ki67 method alone with all p-values < 0.05 (Table 4.7). The dual stain methods were not significantly different from one another for difference (variation) around the mean KPI % estimate with all p-values > 0.05 (Table 4.7).

Table 4. 9. Analysis of KPI by location of the specimen in the GI tract.

Variable	Mean KPI % (all staining methods)	Mean KPI % Ki67-only	Mean KPI % Syn-Ki67	Mean KPI % CgA-Ki67
Upper tract (11)	1.77	0.55	0.30	0.25
Lower tract (8)	0.90	0.70	0.38	0.32

Pairwise t-test comparison of deviation between mean KPI % and each KPI % method (n=11/8)

	Location of Specimen in the GI tract	
Variable	**Upper**	**Lower**
Ki67-only vs Syn-Ki67	p<0.001	p=0.002
Ki67-only vs CgA-Ki67	p=0.003	p=0.002
Syn-Ki67 vs CgA-Ki67	p=0.578	p=0.435

Upper GI tract specimens (n=11) returned a higher mean KPI % (1.77) than Lower GI tract specimens (mean KPI % 0.90) as shown in Table 4.9. An unpaired 2-tailed students t-test indicated that this difference was not significant (p=0.089). Only 8 lower GI tract specimens were included in pairwise comparisons. In the analysis of the KPI performances of the three staining methods, in relation to GI tract location, the mean differences were higher for the gold standard method (Upper 0.55, Lower 0.70), regardless of the level within the GI tract, in comparison to the dual staining methods Syn-Ki67 (Upper 0.30, Lower 0.38) and CgA-Ki67 (Upper 0.25, Lower 0.32). Like results from the full dataset, the Syn-Ki67 and CgA-Ki67 methods were significantly less variable than the Ki67-only staining method with all p-values <0.05 (Table 4.9). The dual stain methods were not significantly different from one another for difference (variation) around the mean KPI % estimate with all p-values > 0.05 (Table 4.9).

It was also observed that Case No. 19, the grade 2 tumour, did not cause the two n=11 data sets (biopsy only, GI tract) to fail the test for normality (Kolgorov Smirnoff test). The smaller datasets allowed the KPI % test performance comparisons to be repeated, showing again the greater concordance and precision of the dual stain methods (Tables 4.8 and 4.9). This prompted the raw data on KPI % for biopsy versus resection and upper versus lower GI tract to be re-examined using unpaired t-tests.

Table 4. 10. KPI scores by Method, Specimen Type and Specimen Level.

Specimen type	Biopsy (n=11)	Resection (n=9)	t-test	t-test (omit #19)
Ki67-only	1.191	1.511	p = 0.529	p = 0.007
Syn-Ki67 DS	1.982	0.744	p = 0.038	p = 0.029
CgA-Ki67 DS*	2.11	0.733	p = 0.013	p = 0.003

Specimen type	Upper GI (n=11)	Lower GI (n=9)	t-test	t-test (omit #19)
Ki67-only	1.391	1.267	p = 0.808	p = 0.381
Syn-Ki67 DS	1.991	0.733	p = 0.034	p = 0.024
CgA-Ki67 DS**	1.936	0.8	p = 0.050	p = 0.030

**There were only 10 values in the dataset tested for Biopsy specimen CgA-Ki67.*

***There were only 8 values in the dataset tested for Lower GI tract CgA-Ki67.*

It was observed that the average KPI % for biopsy derived specimens were higher than for resection derived specimens using the dual stain methods (Table 4.10). This was significant at the 5% level with all p-values <0.05. The opposite trend was observed for Ki67-only alone testing with the magnitude of the difference lower (1.191 versus 1.511) and not significant (p=0.529). For 'Ki67-only', the student t-test statistic was very sensitive to the removal of the grade 2 tumour, returning a significant difference in the opposite direction from the dual stain methods when omitted (p=0.007). In contrast, the dual stain methods showed >2 fold difference and a slightly more significant result in the same direction when #19 was removed from the analysis, presumably because the variance around the test means for biopsies was reduced.

The average KPI % for Upper GI tract derived specimens was higher than for lower GI tract derived specimens using both the Ki67-only approach and the dual stain method (Table 4.10). The difference trend for the Ki67-only method was with a much lower magnitude (1.391 versus 1.267) and not significant (p=0.808). In contrast, the KPI % for the Upper GI tract was > 2-fold higher that lower GI tract values for each dual stain method and all p-values were ≤ 0.05 of the difference lower. The student t-test statistic was again sensitive to the removal of the grade 2 tumour, returning lower p-values when omitted, due to reduced variance around the test means for lower GI tract specimens.

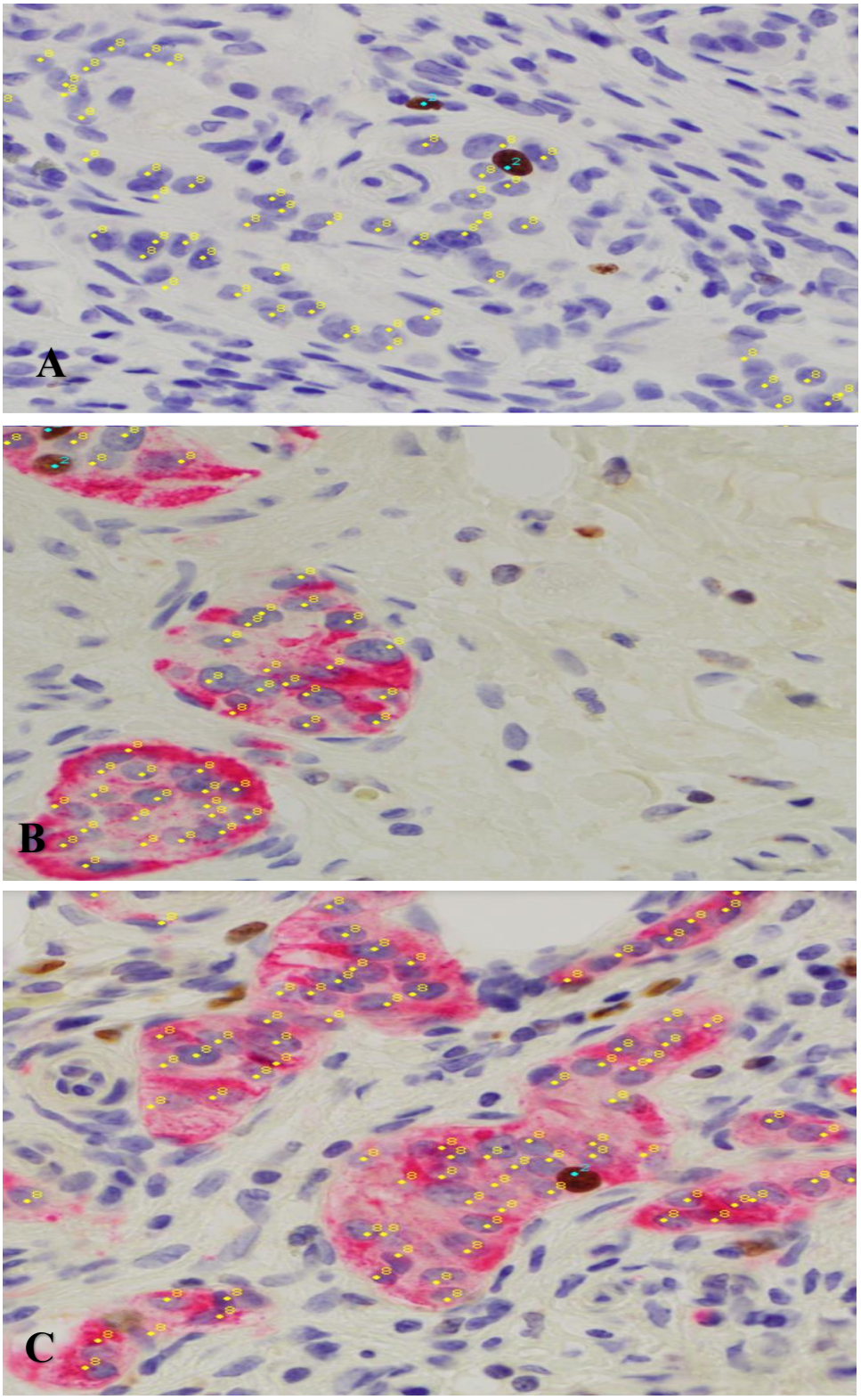

Figure 4. 6. Comparison of the three staining methods used to count tumour and non-tumour cells.

Magnification 200x. Panel A- Ki67-only stain; Panel B- Synaptophysin-Ki67; Panel C-Chromogranin A-Ki67. Number 8 in all images represent the non-tumour cells and the number 2 hovering over the brown dot is pointing toward the tumour cells marked by the Ki67 marker.

4.4 Discussion

The findings reported here for double stain comparisons with the Ki67 'gold standard' clearly show the potential benefit of double stains to determine the KPI accurately. It is uncontested that the double stain methods to obtain a Ki67 proliferative index take the pathologists less time to perform. There is also strong evidence that together with a time saving, the dual stain methods are more accurate and precise. This evidence comes from comparing the performance of each KPI determination method with the average KPI value assigned for each individual case. In this scenario, the variability of both dual stain methods, the difference from the mean KPI, was lower than the variability of the gold standard Ki67-only. The only counter argument to accepting the benefit of a dual stain approach at face value comes from evaluation of the statistical approach and assumptions. The pathologist/s reading the slides varied and it is therefore difficult to 100% exclude the possibility of systematic bias. There is not a 100% guarantee that each estimate of KPI % is independent. The dual stain methods use a more similar protocol to one another than the Ki67-only staining method. However, the intensity of DAB chromogen showing the marker of interest (Ki67) and its contrast with background counterstaining was optimized for all three methods independently. The weight of evidence supporting real benefits for dual-stain estimation of KPI % remains very strong.

The benefit of dual staining is not fully surprising or counter intuitive. The evidence for a genuine boost to accuracy comes from difficulty which arises due to the presence and abundance of background stromal lymphocytes and entrapped non-neoplastic glands which often contain proliferating cells and can easily be mistaken as tumour cells. The delicate vascular network characteristic of neuroendocrine tumours also contains a subset of proliferating cells (10, 40). These non-neoplastic cells can be difficult to distinguish from tumour cells when stained with the gold standard 'Ki67-only' stain and, if mistakenly counted, can artificially elevate the proliferative index. This would also potentially lead to more aggressive treatment option than ideal. Conversely, if the proliferating tumour cells are identified as non-tumour cells then an incorrect negative label applied to tumour cells provides a false negative and underrepresentation of true tumour proliferation could be fatal for the patients (9, 10). This presents not so much as an incorrect diagnosis as an inconsistent assessment of severity and prognosis, given fairly narrow lines of distinction between grade 1 and grade 2 well-differentiated tumours.

It was therefore the aim of this research to examine the impact dual immunohistochemical (IHC) stains (combination of Synaptophysin-Ki67 and of Chromogranin A-Ki67) can have in improving the overall grading of well-differentiated gastrointestinal neuroendocrine tumours (WDGI NETs) by making the counting of the Ki67 tumour cells easier first. The concept of dual (or double) staining methods has been very well established but its application in neuroendocrine neoplasms is limited. Since a dual stain approach is not an expensive method to try, and there are other existent duals stain being used we reasoned there was sufficient expertise and experience to develop and apply these concepts to WDGI NETs. When looking for ways to improve precisions or methods in diagnostic laboratory, costs, workload and time are important factors to take into considerations. The double staining method would introduce a cost in terms of laboratory processing and consumables but had strong potential to increase diagnostic precision and reduce workload for pathologists, measured as KPI assessment time savings. Feedback from reading pathologists regarding the double stains in trials has been positive although there is likely no great urgency for the lab to change its laboratory procedures or modify the pathologists approach to KPI assessment.

It is a common practice to assess the reproducibility of a new method/ assay by evaluating its concordance or agreement to the current assay or method which is often referred to as the 'gold standard'. In many regards this is setting the bar low when available literature suggests the superiority of a dual stain method approach. However, there can easily be technical hurdles with implementation and optimisation of new methods. Therefore, a 'gold standard', with all the existent procedural familiarity, is not to be challenged lightly in diagnostic Anatomical Pathology departments using time poor pathologists in the evaluation process. In this study, we both optimize and validate new dual stains, and confirm the reproducibility and reliability of the two proposed methods (Syn-Ki67 and CgA-Ki67) by comparing the results they each produced (Ki67 proliferative index in percentage) to those of the gold standard 'Ki67-only' stain. Our findings indicated that the two proposed staining methods can reproduce the same results as those obtained by the gold standard in the assessment of KPI but also in the grading of the tumour. This outcome is based on the statistical analysis of the tumour grade concordance in Figure 4.5 where all three of the staining methods strongly agreed in the grading of 19 of the 20 cases (Grade 1: $\kappa = 1$, $p < 0.001$, Strong agreement) and 1 of 20 cases (Grade 2: $\kappa = 1$, $p < 0.001$, Strong agreement). This is a very important finding and telling us that, diagnostically, on well differentiated GI NETs all methods can discriminate between the lower tumour grades with accuracy. It is also indicated that the reliability of the proposed staining methods can be used

on their own if not used alongside the gold standard method. Although there was interest in the original KPI and grade of each of the cases used in this study, leading pathologist involved in this research instructed us not to make further comparisons. New results (if different) may conflict with the prior reported diagnosis and potentially influence views on patient outcomes. It was considered preferable to look at the performance of reporting pathologists in a new study going forward and with no implications for patient well-being. As a result, new opportunities arise for a future prospective study utilizing our double staining approach. Human ethics approval as well as statistician input would be desirable / necessary under most of these future focussed circumstances, especially if patient outcomes and evolving epidemiology of GI NETS become factored into the design.

This study also had an interest in investigating if different specimen types and tumours from different parts/levels of the GI tract can influence or affect the performance of each of the three staining methods assessing KPI. There were several potential reasons to why it was worthwhile investigating this. Resection specimens come from parts of the body where a complete excision is more possible, for example appendix or colon and perhaps the stomach. Biopsies come from parts of the body from which a complete excision (with margin confidence) is more difficult. Surgery on the liver or pancreas is more challenging in terms of complications. Furthermore, resected specimens provide more material for the pathologist and is likely to be more representative. i.e. if > 1 block and slide more chances to find a hot spot or replicated region of interest. In contrast biopsies have less material represented, potentially more artefacts, crush thermal etc and perhaps not all biopsies give same level of artefact. i.e. core difficult to grasp. In order to investigate this, the twenty cases used in this study were divided into resection or biopsy specimen types. All biopsies including the core biopsies were treated as general endoscopic biopsies regardless of what they are. Core biopsies used in this research were obtained via percutaneous liver biopsy using a fine needle endoscopically. It is known to be the least invasive method to obtain a liver biopsy in contrast to transvenous liver biopsy and a surgical liver biopsy. Non-core biopsies used in this research were retrieved using one of the following endoscopy-assisted procedures; endoscopic submucosal dissection; cold forceps biopsy; endoscopy resection and colonoscopy in which polyp is either removed completely or partially. Resection specimens such as appendix in this study were obtained via any of the following surgical procedures: laparoscopy appendicectomy; right hemicolectomy and wedge resection of the stomach. Biopsy specimens are chosen over resection specimens for a variety of clinical reasons and this information was not considered or consented in the research

objective. Our study found that biopsy specimen types had a higher KPI % when assessed using the dual stain methods. Several lines of evidence suggest that this is likely to be a true result based on the overall concordance and precision of dual staining methods. The explanation for this could very well be clinically based, in that biopsies are used for more difficult to access and poorly operable tumours such as might reside in the stomach, pancreas and liver. These tumours might be diagnosed at a later stage and with more aggressive proliferation profiles. In contrast, more easily resectable tumours are likely associated with the rectum and appendix and perhaps diagnosed earlier. Another very significant consideration is the fact that biopsies are more likely to be associated with collection and processing artefacts. Crushing, cautery, and any rough handling of biopsies can introduce damage to tissues and this damage can manifest by changing how tissues perform under diagnostic processing and testing. Folding of tissues, focal areas of damage and reduced options for selecting the region of interest, due to a lower volume of diagnostic material, could all be factors in why there was a demonstrably higher KPI % in biopsy specimens compared with resected specimens. While an exact explanation is not in hand, the observation is recorded and will be of interest to pathologists, oncologists and histotechnologists working in this field.

There was also interest in comparing upper GI tract tumours with lower GI tract tumours because tumour location has a bearing on the outcome of a case, regardless of grade. This is because full surgical resection is possible for certain location, for example appendix, more readily than tumours associated with the GI tract but beyond digestive mucosal surfaces. In other words, upper GI tract NETs, for example from the liver or pancreas, can invade toward poorly and inoperative sites as well as being more difficult to access (or resect) for diagnostic purposes. Our study showed that upper GI tract NETs had a significantly higher KPI % that lower GI tract NETs using the dual stain methods. This is likely to reflect a true finding, corroborated by the direction of the difference for the KI67-only stain method, even though this trend was not significant. Again, the clinical behaviour of tumours from upper versus lower GI tract and the clinical approach may have been influential in observing these differences. For example, a large but grade 1 space-occupying tumour close to important structures is problematic for different reasons than a small grade 3 tumour with a higher metastatic potential. The test dataset had physical metrics on the tumours and a variety of sizes were represented. No relationship between size and KPI % was observed (data not presented). In the same way that the higher KPI % seen with biopsies did not have a simple explanation, the reason for higher KPI % for the upper GI tract tumours was not resolved. We did not test for a relationship

(correlation) between biopsy status and level within GI tract and how this might explain KPI % estimations for a variety of reasons. The first reason was scope. This study is about test validation of new methods, demonstrating potential superiority of dual stains and the reasons why this is plausible. The second reason was a concern about the rigor and independence of the KPI % evaluation. Effectively a single metric was available for each method validated. Simple statistical approaches were chosen to show test effectiveness. In contrast, to explain differences (albeit significant) and some opposing trends in the data about biopsy and level within GI tracts effects further replication of KPI % using standardised methodology would be recommended, as would further input from data analysts and the consideration of more complex statistical models. None-the-less, these observations are worthy of note and further investigation into the future.

The mean differences in the amount of time taken by the reading pathologists to complete assessment of the Ki67 proliferative index for each of the staining method were calculated to assess efficiency of each method. Both double staining methods Syn-Ki67 and CgA-Ki67 were faster, and it took less than half of the time required by the gold standard to complete an assessment. These differences in means between the methods were shown to be highly significant in our statistical analysis. Not only is this result consistent with the literature (9, 39) but it also supported our hypothesis that the double staining method to make tumour cells more visible to count and require less time for pathologists to grade WDGI NETs cases. Unless distracted, pathologists spend less time deciding if a proliferating cell is part of the tumour cell or non-tumour cell population when there is a second marker assisting to define tumour cells.

Our most interesting finding is the potential for the dual stains to outperform the gold standard Ki67-only stain. The disparity between the mean KPI score across all three methods was highest for Ki67-only stain, followed by Syn-Ki67 and CgA-Ki67. Our methodology is a little unorthodox from the perspective of potential discrepancies in calculating the KPI mean. We cannot assume each assessment of KPI used to derive the means is independent of observer bias. Our initial findings are relatively easy to investigate further. One approach is prospective, in that these tumours are diagnosed on an ongoing basis. It would be feasible for an interest group to follow the performance of the dual stain and gold standard as a comparison going forward. Furthermore, the differences in performance of each staining methods assessing the KPI could have been done better by also studying the concordance between different readers (or different pathologists reading the same specimens but reading slides stained using the different KPI methods). For future study, for example looking at biopsy performance it will be

better to use a larger study sample size. However, for validation (i.e. this study outcome) it will be sufficient to stay with 20 specimens and beneficial (but not obligatory) to send the slides off to be read by a single external reader (or contracts one or two people). Ideally the concept of blinding (anonymizing) samples and presenting specimens in random order is discussed in the methodological approach ahead. If two people can be contracted to independently read slide sets ahead of a comparison, then the gold standard for experimental approach is fulfilled at the same time as testing the gold standard Ki67-only stain method against a competing method.

Some possible limitations of this study may include the use of a relatively small study sample size (i.e.20 cases). However, a great number of optimization and validation studies are performed on a sample size of 20 or fewer, simply because of the investment in technical and pathologist time required to design and execute validation level trials. It was interesting to note that even the subsets of specimens, segregated by specimen type or level, gave good evidence for a performance advantage by the dual stain methods. Boosting the likely concordance of methods and trial success with 20 specimens, was adhering to selection criteria. Had a greater range of GI NET severity been included, total diagnostic agreement (grade-wise) between methods might have been more difficult to obtain. For example, had the intersection of grade two and grade three tumours been selected or if grade one tumours had been excluded. Narrowing the grade range investigated but making 'type of specimen' a variable in the follow-on analysis asked about test performance relative to input material and was informed by pathologist experience of reading around artefacts and reduced volumes of diagnostic material. As diagnostic procedures linked to surgery become less invasive and 'key hole' so does the amount of assessable tumour material decrease. Added to the greater likelihood of specimen damage due to collection or processing. Making diagnoses from increasingly small amounts of material places more pressure and responsibility on pathologists. In this scenario, the accuracy of tests is obliged to increase if improvements can be identified. This study was able to document increased precision and accuracy but was not able to comment with authority about biopsies as an increasing proportion of diagnostic submissions. Availability of pathologists was a significant limitation in this study and required much planning to fit research requests into their busy schedule. For instance, minor adjustments needed for the research method/evaluation (change to scoring system utilized in the study) was not possible as pathologists were unavailable to do a re-evaluation of the slides in Part 1 of the research due to either busy to their work commitment or were away on leave.

This study might have been benefitted from further buy-in from pathologists in terms of reviewing complete data sets and having single reader scoring on selected subsets to be compared. One brief from the outset of the trial was to limit the KPI scoring time commitment to validation by a workforce with time pressure and turnaround time demands by clinicians. A second brief from the outset was that the study was not to evaluate pathologists scoring concordance. This variable scope of pathologists input was also partially evident in the lack of comments from the reading pathologists reading any difficult cases they might have encountered in their assessment. Recuts and re-stains were not requested, and no untoward issues were reported over the KPI percentage assessment and time recorded. The study was not able to assume or report that a single pathologist provided every KPI assessment in the Ki67-only assessments and Ki67 dual stains or that biopsies versus resections had been evaluated by a single person. This has not prevented extremely useful information from having been gathered and interpreted. However, it does demand some caution in the interpretation as well. We believe that with more up-front attention to design, and with a different execution approach to the KPI reading we might have corroborated the observed difference and trends more strongly. Alternatively, our preliminary findings could be challenged. Either way, findings that support both validation and reportable differences, with a single metric for each GI NET trial, strengthens our confidence in the pathologist team and methodology for working up diagnostic improvements at Middlemore Hospital Histology.

4.5 Conclusion

In conclusion, we successfully validated reproducibility and reliability of each of the two double staining techniques: Synaptophysin-Ki67 and Chromogranin A-Ki67 to be used either alongside the gold standard Ki67-only stain or on their own for the assessment of KPIs in the grading of well-differentiated gastrointestinal neuroendocrine tumours (WDGNETs). This conclusion was drawn from the outcome of the statistical analysis of the experiments conducted above. All three of the staining methods (Ki67-only stain, Syn-Ki67 and CgA-Ki67) not only agreed in the KPI assessments of the 20 selected cases in this study but they were also strongly agreed (κ-value of 1, very strong agreement) on the grading of all 20 tumours. The intercorrelation coefficient (ICC) between the gold standard Ki67-only staining method and each of the proposed double staining methods were strong and the correlations were statistically significant. The very high ICC of 0.982 between the two double staining methods also strongly supported reliability and reproducibility of each method especially the newly trialled CgA-

Ki67 staining method. It also showed the reliability of both double staining methods and it is proposed that each could be used on their own if required.

Our study also found that specimen types (biopsy or resection) and location of the tumour specimens (upper vs lower GI tract) in the gastrointestinal system (upper or lower gastrointestinal tract) does have some degree of influence or impact on the performance of each method in the assessment of KPI. Drawn from the statistical analysis above (Table 4.6 to 4.10), the KPI % assessment results were significantly higher for the biopsy subset stained using the dual staining methods (Syn-Ki67 and CgA-Ki67) when compared to the resection subset. The Ki67-only staining method could not replicate this finding. The KPI % assessment results were also significantly higher for the upper GI tract subset when stained with the dual staining methods in contrast to the lower GI tract subset. More research would be required to replicate these findings and better understand the implications to these differences.

Furthermore, the double staining methods are also more efficient than the Ki67-only staining method showing that it can take less time to complete a KPI assessment. This makes the double staining methods better suited into our laboratory and fast paced diagnostic laboratory setting around the country. It is possible that the efficiency of the double staining methods is due to their ability to highlight tumour cells which in turn helps prevent pathologists from wasting time in undercounting tumour cells in biopsies and overcounting tumour cells in resection cases. This research will encourage the use of dual stains to improve visualization of tumour cells making it easier to assess the Ki67 proliferative index in well-differentiated gastrointestinal neuroendocrine tumours.

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6.0 Appendix

6.1 Appendix 1

Number of HPF required for 10 mm² using microscopes with different field diameter p10.

FIELD DIAMETER (mm)	AREA (mm²)	NUMBER OF HPF FOR 10 mm²
0.40	0.125	80
0.41	0.132	75
0.42	0.139	70
0.43	0.145	69
0.44	0.152	65
0.45	0.159	63
0.46	0.166	60
0.47	0.173	58
0.48	0.181	55
0.49	0.189	53
0.50	0.196	50
0.51	0.204	49
0.52	0.212	47
0.53	0.221	45
0.54	0.229	44
0.55	0.238	42
0.56	0.246	41
0.57	0.255	39
0.58	0.264	38
0.59	0.273	37
0.60	0.283	35
0.61	0.292	34
0.62	0.302	33
0.63	0.312	32
0.64	0.322	31

6.2 Appendix 2

Monoclonal Mouse Anti-Human Ki-67 Antigen
Clone MIB-1

Code M7240

ENGLISH

Intended use

For in vitro diagnostic use.

Monoclonal Mouse Anti-Human Ki-67 Antigen, Clone MIB-1, is intended for use in immunohistochemistry (IHC). The antibody is useful for the identification of the Ki-67 antigen in normal and neoplastic cells (1). Differential classification of tumors is aided by the results from a panel of antibodies. The clinical interpretation of any staining or its absence should be complemented by morphological studies using proper controls and should be evaluated within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist. This antibody is intended to be used after the primary diagnosis of tumor has been made by conventional histopathology using nonimmunologic histochemical stains.

Summary and explanation

The Ki-67 antigen is a nuclear protein, which is defined by its reactivity with monoclonal antibody from the Ki-67 clone (2). Two isoforms of 345 and 395 kDa have been identified (3). The Ki-67 antigen is preferentially expressed during all active phases of the cell cycle (G_1, S, G_2 and M-phases), but it is absent in resting cells (G_0-phase) (2). During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The antigen is rapidly degraded as the cell enters the non-proliferative state (4), and there appears to be no expression of Ki-67 during DNA repair processes (5).

Refer to *Dako General Instructions for Immunohistochemical Staining* or the detection system instructions of IHC procedures for: Principle of Procedure; Materials Required, Not Supplied; Storage, Specimen Preparation; Staining Procedure; Quality Control; Troubleshooting; Interpretation of Staining; General Limitations.

Reagent provided

Monoclonal mouse antibody provided in liquid form as cell culture supernatant dialysed against 0.05 mol/L Tris-HCl, 1% bovine serum albumin, pH 7.2, and containing 15 mmol/L NaN_3.

Clone: MIB-1 (6). Isotype: IgG1, kappa.
Mouse Ig concentration: see label on vial.

The protein concentration between lots may vary without influencing the optimal dilution. The titer of each individual lot is compared and adjusted to a reference lot to ensure a consistent immunohistochemical staining performance from lot-to-lot.

Immunogen

Human recombinant peptide corresponding to a 1002 bp Ki-67 cDNA fragment (6).

Specificity

In Western blotting of lysates of the multiple myeloma cell line, IM-9, the MIB-1 antibody labels bands of 345 and 395 kDa, identical to the bands labelled by the original Ki-67 antibody. Furthermore, Western blotting and competitive binding experiments clearly demonstrate that MIB-1, like the original Ki-67 antibody, reacts with an epitope encoded by a 66 bp repetitive element in the Ki-67 gene. In immunohistochemistry, the MIB-1 and the Ki-67 antibodies provide identical staining patterns on serial tonsillar frozen sections (6). The MIB-1 antibody recognizes native Ki-67 antigen and recombinant fragments of the Ki-67 molecule (6).

Precautions

1. For in vitro diagnostic use.
2. For professional users.
3. This product contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.
4. As with any product derived from biological sources, proper handling procedures should be used.
5. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
6. Unused solution should be disposed of according to local, State and Federal regulations.

Storage

Store at 2-8 °C. Do not use after expiration date stamped on vial. If reagents are stored under any conditions other than those specified, the conditions must be verified by the user. There are no obvious signs to indicate instability of this product. Therefore, positive and negative controls should be run simultaneously with patient specimens. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the antibody is suspected, contact Dako Technical Support.

Specimen preparation

Paraffin sections: The antibody can be used for labeling paraffin-embedded tissue sections fixed in formalin.

Pre-treatment: Pre-treatment of formalin-fixed, paraffin-embedded tissue sections with heat-induced epitope retrieval (HIER) is required. Optimal results are obtained by pretreating deparaffinized tissues with HIER using diluted Dako Target Retrieval Solution, Low pH (10x) (Code S1699) or diluted EnVision FLEX Target Retrieval Solution, Low pH (50x) (Code K8005) for 20 minutes. The tissue sections should not dry out during the treatment or during the following immunohistochemical staining procedure.

Staining procedure

These are guidelines only. Optimal conditions may vary depending on specimen type and preparation method, and should be validated individually by each laboratory. The performance of this antibody should be established by the user when utilized with other manual staining systems or automated platforms.

Dilution: Monoclonal Mouse Anti-Human Ki-67 Antigen, Code M7240, may be used at a dilution range of 1:75-1:150 when applied on formalin-fixed, paraffin-embedded sections of human tonsil or human intestinal mucosa and using 20 minutes heat-induced epitope retrieval in Target Retrieval Solution, Low pH (Code S1699/K8005), and 20 minutes incubation at room temperature with the primary antibody. The recommended negative control is Dako Mouse IgG1, Code X0931, diluted to the same mouse IgG concentration as the primary antibody. Unless the stability of the diluted antibody and negative control has been established in the actual staining procedure, it is recommended to dilute these reagents immediately before use, or dilute in Dako Antibody Diluent, Code S0809.

Quality Control: Positive and negative control tissues as well as negative control reagent should be run simultaneously using the same protocol as the patient specimens.

Visualization: The recommended visualization system is EnVision FLEX, High pH (Code K8000/K8010) using a 20 minutes incubation at room temperature. Follow the procedure enclosed with the visualization kit.

Note: Use diluted Dako Target Retrieval Solution, **Low pH** (10x) (Code S1699) or diluted EnVision FLEX Target Retrieval Solution, **Low pH** (50x) (Code K8005) for HIER.

Product-specific limitations

Occasional labelling of tissue components in vessel walls and pancreatic stroma has been observed in immunohistochemistry.

Staining interpretation

Cells labeled by the antibody display a nuclear staining pattern except in mitotic cells, where the chromosomes and the cytoplasm are labeled.

6.3 Appendix 3

CELL MARQUE™
a SIGMA-ALDRICH company

Synaptophysin (MRQ-40) Rabbit Monoclonal Antibody

For In Vitro Diagnostic Use (IVD)

Product Identification

REF	Description
336R-94	0.1 mL concentrate
336R-95	0.5 mL concentrate
336R-96	1.0 mL concentrate
336R-97	1.0 mL predilute ready-to-use
336R-98	7.0 mL predilute ready-to-use

Symbol Definitions

KEY-CODE	keycode
P	predilute
C	concentrate
A	ascites
E	serum
S	supernatant
DIL	concentrate dilution range

Intended Use

This antibody is intended for *in vitro* diagnostic (IVD) use.

Synaptophysin (MRQ-40) Rabbit Monoclonal Primary Antibody is intended for laboratory use in the detection of the Synaptophysin glycoprotein in formalin-fixed, paraffin-embedded human tissue stained in qualitative immunohistochemistry (IHC) testing.

The results using this product should be interpreted by a qualified pathologist in conjunction with the patient's relevant clinical history, other diagnostic tests and proper controls.

Summary and Explanation

Anti-synaptophysin reacts with neuroendocrine cells of human adrenal medulla, pituitary, thyroid, lung, pancreas, and gastrointestinal mucosa. Positive staining is seen in neurons of the brain. This antibody identifies normal neuroendocrine cells and neuroendocrine neoplasms.[1-5] Diffuse, finely granular, cytoplasmic staining is observed, which probably correlates with the distribution of the antigen within neurosecretory vesicles. Anti-synaptophysin is an independent, broad-range marker of neural and neuroendocrine differentiation.[1-9]

Principles and Procedures

The stated primary antibody may be used as the primary antibody for immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections. In general, immunohisto-chemical staining in conjunction with a HRP or Alk Phos linked detection system allows the visualization of antigens via the sequential application of a specific antibody (primary antibody) to the antigen, a secondary antibody (link antibody) to the primary antibody, an enzyme complex and a chromogenic substrate with interposed washing steps. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site. The specimen may then be counterstained and a coverslip applied. Results are interpreted using a light microscope.

Materials and Methods

Reagents Provided

Product Composition	
Predilute: diluted in	Tris Buffer, pH 7.3-7.7, with 1% BSA and <0.1% Sodium Azide
Concentrate: diluted in	Tris Buffer, pH 7.3-7.7, with 1% BSA and <0.1% Sodium Azide
Host	Rabbit
Isotype	IgG_1
Recommended working dilution range	1:100-1:500
Source	Supernatant

See product label for lot specific information for the following:

1. Antibody immunoglobulin concentration
2. Source details

Reconstitution, Mixing, Dilution, Titration

Prediluted antibody is ready-to-use and optimized for staining. No reconstitution, mixing, dilution, or titration is required. The concentrated antibody is optimized to be diluted to within the dilution range using Cell Marque Diamond Diluent.

Materials and Reagents Needed But Not Provided

The following reagents and materials may be required for staining but are not provided with the primary antibody:

1. Positive and negative control tissue
2. Microscope slides, positively charged
3. Drying oven capable of maintaining a temperature of 53-65°C
4. Staining jars or baths

6.4 Appendix 4

invitrogen

Catalog # MA5-42925

Chromogranin A Recombinant Rabbit Monoclonal Antibody (2N5Q4)

Product Details	
Size	100 µL
Species Reactivity	Human, Mouse, Rat
Host/Isotype	Rabbit / IgG
Expression system	HEK293 cells
Class	Recombinant Monoclonal
Type	Antibody
Clone	2N5Q4
Conjugate	Unconjugated
Immunogen	A synthetic peptide corresponding to a sequence within amino acids 1-100 of human Chromogranin A (P10645).
Form	Liquid
Concentration	0.5 mg/mL
Purification	Affinity Chromatography
Storage buffer	PBS, pH 7.3, with 0.05% BSA, 50% glycerol
Contains	0.02% sodium azide
Storage conditions	-20° C, Avoid Freeze/Thaw Cycles
RRID	AB_2912066

Applications	Tested Dilution	Publications
Western Blot (WB)	1:500-1:1,000	-
Immunohistochemistry (Paraffin) (IHC (P))	1:50-1:200	-
Immunocytochemistry (ICC/IF)	1:50-1:200	-

Product Specific Information

Positive test controls include: PC-12, SH-SY5Y.

Immunogen sequence: MRSAAVLALL LCAGQVTALP VNSPMNKGDT EVMKCIVEVI SDTLSKPSPM PVSQECFETL RGDERILSIL RHQNLLKELQ DLALQGAKER AHQQKKHSGF

6.5 Appendix 5

BOND Polymer Refine Detection

Catalog No: DS9800

For Professional Use Only

Intended Purpose

For *in vitro* diagnostic use.

BOND Polymer Refine Detection is a biotin-free, polymeric horseradish peroxidase (HRP)-linker antibody conjugate system intended for the visualization of tissue-bound mouse IgG, mouse IgM and rabbit primary antibodies. It is intended for target visualization by immunohistochemistry (IHC) or chromogenic *in situ* hybridization (CISH) in sections of formalin fixed, paraffin-embedded tissue using the automated BOND-MAX or BOND-III systems.

The clinical interpretation of any staining or its absence should be complemented by morphological studies and proper controls should be evaluated within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist.

BOND Polymer Refine Detection is intended to be used with other devices for visualization of staining and as such, qualitative or semi-quantitative diagnostic function including specific disease indication and intended use population is described within the associated device labelling as required for that device.

Summary and Explanation

Immunohistochemical (IHC) staining techniques allow for the visualization of antigens via the sequential application of a specific antibody to the antigen (primary antibody), a secondary antibody to the primary antibody and an enzyme complex with a chromogenic substrate with interposed washing steps.

BOND Polymer Refine Detection utilizes a novel controlled polymerization technology to prepare polymeric HRP-linker antibody conjugates. The detection system avoids the use of streptavidin and biotin, and therefore eliminates non-specific staining as a result of endogenous biotin.

BOND Polymer Refine Detection works as follows:

- The specimen is incubated with hydrogen peroxide to quench endogenous peroxidase activity.
- A user-supplied specific primary antibody is applied.
- Post Primary IgG linker reagent localizes mouse antibodies.
- Poly-HRP IgG reagent localizes rabbit antibodies.
- The substrate chromogen, 3,3'-Diaminobenzidine tetrahydrochloride hydrate (DAB), visualizes the complex via a brown precipitate.
- Hematoxylin (blue) counterstaining allows the visualization of cell nuclei.

Using BOND Polymer Refine Detection in combination with the BOND-MAX and BOND-III automated systems reduces the possibility of human error and inherent variability resulting from individual reagent dilution, manual pipetting and reagent application.

Reagents Provided

Reagents sufficient for 200–300 tests

1. Peroxide Block (30 mL) 3–4% (v/v) Hydrogen peroxide.
2. Post Primary (30 mL) Rabbit anti mouse IgG (<10 µg/mL) in 10% (v/v) animal serum in tris-buffered saline/0.1% ProClin™ 950.
3. Polymer (30 mL) Anti-rabbit Poly-HRP-IgG (<25 µg/mL) containing 10% (v/v) animal serum in tris-buffered saline/0.1% ProClin™ 950.
4. DAB Part 1 (2.4 mL) 66 mM 3,3'-Diaminobenzidine tetrahydrochloride hydrate, in a stabilizer solution.
5. DAB Part B (30 mL) ≤0.1% (v/v) Hydrogen Peroxide in a stabilizer solution.
6. DAB Part B (30 mL) ≤0.1% (v/v) Hydrogen Peroxide in a stabilizer solution.
7. Hematoxylin (30 mL) <0.1% Hematoxylin.

Dilution and Mixing

BOND Polymer Refine Detection is optimized for use on the automated BOND-MAX or BOND-III systems. Reconstitution, mixing, dilution, or titration of these reagents is not required.

Materials Required But Not Provided

BOND Dewax Solution (Catalog No. AR9222)

BOND Epitope Retrieval Solution (Catalog No. AR9961, AR9640 or AR9551)

BOND Wash Solution 10X Concentrate (Catalog No. AR9590)

BOND-MAX system or BOND-III system

Refer to "Using BOND Reagents" in your BOND user documentation for a complete list of materials required for specimen treatment and immunohistochemical staining using the BOND system (includes BOND-MAX system and BOND-III system).

Storage and Stability

Store at 2–8 °C. Do not freeze. Do not use after the expiration date indicated on the tray handle label. Return to 2–8 °C immediately after use.

There are no obvious signs to indicate instability of this product, therefore positive and negative controls should be run simultaneously with unknown specimens (refer to "Quality Control" in the "Using BOND Reagents" section of your BOND user documentation).

If unexpected staining is observed that cannot be explained by variations in laboratory procedures, and a problem with the detection system is suspected, contact your local distributor or the regional office of Leica Biosystems immediately.

Storage conditions other than those specified above must be verified by the user[1].

6.6 Appendix 6

ChromoPlex 1 Dual Detection for BOND - 50 Test

Catalog No: DS9665

For Professional Use Only

Intended Purpose

For *in vitro* diagnostic use.

ChromoPlex 1 Dual Detection is a biotin-free, polymeric horseradish peroxidase (HRP)-linker and polymeric alkaline phosphatase (AP)-linker antibody conjugate system for the visualization of tissue-bound mouse IgG and rabbit primary antibodies. It is intended for target visualization by immunohistochemistry (IHC) in sections of formalin fixed, paraffin-embedded tissue using the automated BOND-MAX or BOND-III systems.

The clinical interpretation of any staining or its absence should be complemented by morphological studies and proper controls should be evaluated within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist.

ChromoPlex 1 Dual Detection is intended to be used with other devices for visualization of staining and as such, qualitative or semi-quantitative diagnostic function including specific disease indication and intended use population is described within the associated device labelling as required for that device.

Summary and Explanation

Immunohistochemical (IHC) staining techniques allow for the visualization of antigens via the sequential application of a specific antibody to the antigen (primary antibody), a secondary antibody to the primary antibody and an enzyme complex with a chromogenic substrate with interposed washing steps.

ChromoPlex 1 Dual Detection utilizes a novel controlled polymerization technology to prepare polymeric HRP-linker and AP-linker antibody conjugates. The detection system avoids the use of streptavidin and biotin, and therefore eliminates non-specific staining as a result of endogenous biotin.

ChromoPlex 1 Dual Detection works as follows:

- The specimen is incubated with hydrogen peroxide to quench endogenous peroxidase activity.
- A user-supplied specific primary mouse and rabbit antibody cocktail is applied.
- Poly-HRP IgG reagent localizes mouse antibodies.
- Poly-AP IgG reagent localizes rabbit antibodies.
- The first substrate chromogen, 3,3'-Diaminobenizidine tetrahydrochloride hydrate (DAB), visualizes mouse antibodies via a brown precipitate.
- The second substrate chromogen (Fast Red) visualizes rabbit antibodies via a red precipitate.
- Hematoxylin (blue) counterstaining allows the visualization of cell nuclei.

Using ChromoPlex 1 Dual Detection in combination with the BOND-MAX and BOND-III automated systems reduces the possibility of human error and inherent variability resulting from individual reagent dilution, manual pipetting and reagent application.

Reagents Provided

The reagents provided are sufficient for 25 individual BOND staining runs, or a maximum of 50 slides.

To achieve a maximum of 50 slides from this detection system, slides must be batched in quantities of 2 or greater, per Slide Staining Assembly. Batching in quantities of less than 2 will result in fewer stained slides.

1. Peroxide Block (7.5 mL) 3-4% Hydrogen peroxide.
2. Polymer mHRP (7.5 mL) Poly-HRP anti-mouse containing 10% (v/v) animal serum in Tris-buffered saline and 0.1% ProClin™ 950.
3. Polymer rAP (7.5 mL) Poly-AP anti-rabbit containing 10% (v/v) animal serum in Tris-buffered saline and 0.09% ProClin™ 950.
4. DAB Part 1 (1 mL) 66 mM 3,3'-Diaminobenzidine tetrahydrochloride hydrate in a stabilizer solution.
5. DAB Part B (20 mL) ≤0.1% (v/v) Hydrogen peroxide in a stabilizer solution.
6. Red Part A (5.5 mL) Activator containing 0.5% ProClin™ 950.
7. Red Part B (1.0 mL) Substrate.
8. Red Part C (1.0 mL) Substrate.
9. Red Part D (35 mL) Buffer solution containing 0.5% ProClin™ 950.
10. Hematoxylin DS9665 (7.5 mL) <0.1% Hematoxylin.

Dilution and Mixing

ChromoPlex 1 Dual Detection is optimized for use on the BOND-MAX and BOND-III automated systems.

Reconstitution, mixing, dilution, or titration of these reagents is not required.

Materials Required But Not Provided

BOND Dewax Solution (Catalog No. AR9222)

BOND Epitope Retrieval Solution (Catalog No. AR9961, AR9640 or AR9551)

BOND Wash Solution 10X Concentrate (Catalog No. AR9590)

BOND-MAX system or BOND-III system

Refer to "Using BOND Reagents" in your BOND user documentation for a complete list of materials required for specimen treatment and immunohistochemical staining using the BOND system (includes BOND-MAX system and BOND-III system).