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OPTIMIZATION AND VALIDATION OF FOXP3, BRAFV600E AND MMR IMMUNOHISTOCHEMISTRY STAINING PROTOCOLS

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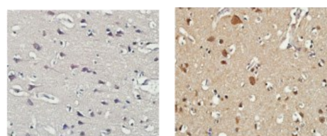
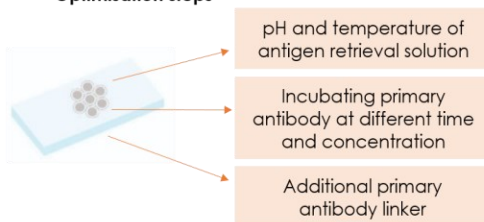
Graphical abstract

Enhancing the IHC staining quality



Low staining signal → High staining signal



Abstract

To ensure consistent and repeatable results, the optimal immunohistochemistry (IHC) staining conditions must be determined when utilising a new antibody. Here, we described how manipulating several technical variables of IHC such as antigen retrieval conditions such as pH and temperature, primary antibody concentration and incubation period could enhance the IHC signals of the targeted antibodies. Mismatch Repair (MMR) proteins such as MutL homolog 1 (MLH1), Mut S homolog 2 (MSH2), Mut S homolog 6 (MSH6), and postmeiotic segregation 2 (PMS2), including B-rapidly accelerated fibrosarcoma with V600E mutation (BRAFV600E) protein and forkhead box P3 (FOXP3) protein, are important for colorectal cancer diagnosis. The IHC staining was performed using different archival tissue controls and commercial antibodies. MLH1 and PMS2 showed higher staining intensity after an overnight incubation at a higher concentration of primary antibody (1:50) compared to BRAFV600E (1:100). The enhanced MSH2 signal was likewise generated at a one-hour incubation period with an equivalent antibody concentration. However, even with a shorter incubation duration of one hour, MSH6 and FOXP3 generated good IHC signals when incubated with primary antibody at a lower antibody concentration of 1:100 and 1:300, respectively. The addition of a primary antibody linker improved the IHC signals for all targeted proteins. In conclusion, when using archival tissues, modifying the aforementioned IHC staining variables produces optimal staining for the MMR, BRAFV600E, and FOXP3 proteins.

Keywords: Immunohistochemistry, optimisation, FOXP3, BRAFV600E, MMR

Abstrak

Untuk memastikan keputusan yang konsisten dan berulang, keadaan pewarnaan imunohistokimia (IHC) yang optimum mesti ditentukan apabila menggunakan antibodi baru. Di sini, kami menerangkan bagaimana memanipulasi beberapa pembolehubah teknikal IHC seperti keadaan pencarian antigen sebagaimana pH dan suhu, kepekatan antibodi primer dan tempoh inkubasi dapat meningkatkan isyarat IHC antibodi yang disasarkan. Protin Pembaikan Tidak Sepadan (MMR) seperti MutL homolog 1 (MLH1), Mut S homolog 2 (MSH2), Mut S homolog 6 (MSH6), dan pengasingan postmeiotic 2 (PMS2), termasuk protin fibrosarcoma dipercepatkan B dengan mutasi V600E (BRAFV600E) dan

protin kotak forkhead P3 (FOXP3), adalah penting untuk diagnosis kanser kolorektal. Pewarnaan IHC dilakukan menggunakan tisu arkib kawalan dan antibodi komersial yang berbeza. MLH1 dan PMS2 menunjukkan intensiti pewarnaan yang lebih tinggi selepas inkubasi semalaman pada kepekatan antibodi primer yang lebih tinggi (1:50) berbanding BRAFV600E (1:100). Peningkatan isyarat MSH2 pula dijana pada tempoh inkubasi satu jam dengan kepekatan antibodi yang setara. Walaubagaimanapun, walaupun dengan tempoh inkubasi yang lebih pendek selama satu jam, MSH6 dan FOXP3 menghasilkan isyarat IHC yang baik apabila disemai dengan antibodi primer pada kepekatan antibodi yang lebih rendah, masing-masing 1:100 dan 1:300. Penambahan penghubung antibodi utama meningkatkan isyarat IHC untuk semua protein yang disasarkan. Kesimpulannya, apabila menggunakan tisu arkib, mengubah suai pembolehubah pewarnaan IHC yang disebutkan di atas menghasilkan pewarnaan optimum untuk protein MMR, BRAFV600E, dan FOXP3.

Kata kunci: Imunohistokimia, pengoptimuman, FOXP3, BRAFV600E, MMR

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1.0 INTRODUCTION

Immunohistochemistry (IHC) offers semi-quantitative information regarding targeted protein expression, distribution and localisation in tissues or cells using antibodies. IHC is one of the most sensitive and specific histochemical procedures as it uses labelled antibodies to locate particular cell and tissue antigens (Figure 1) [1, 2]. Effective antibody and antigen reactions rely primarily on selecting a primary antibody with a specific epitope that is exposed to a variable optimum reaction medium for visible staining while limiting non-specific background signals [3]. IHC staining includes the use of either fresh or archived tissues. Protein antigenicity may be reduced lower in archived tissues than in fresh tissues, depending on how long they have been preserved [4].

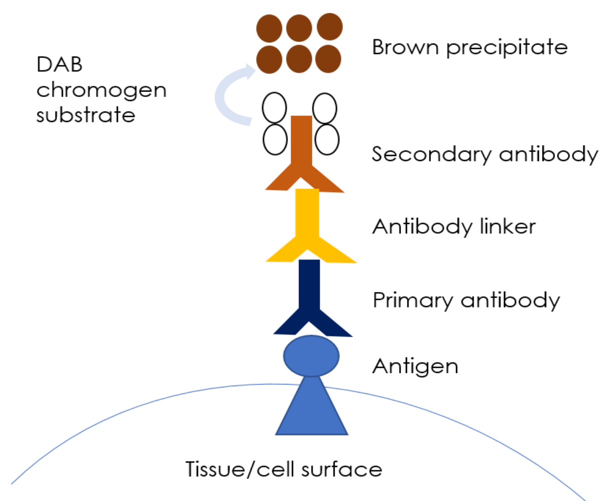

Figure 1 Role of primary antibody, antibody linker and secondary antibody in IHC staining principle

Tissues are usually sent and processed appropriately in the Pathology Laboratory once they are removed from the surgery or collected as a biopsy sample. The histological tissues are subjected to a series of reagents in a stage-wise manner to produce formalin-fixed paraffin-embedded (FFPE) blocks for diagnosis and archived for a maximum of 10 years, depending on the policy of individual facilities [5]. The tissues are archived because they serve as a critical and valuable resource for further diagnostic, future referral and research purposes diagnosis [6-8]. Apart from employing the IHC method on these tissues, DNA and RNA can be extracted from the archival tissues for further molecular testing. Molecular testing such as polymerase chain reaction (PCR), fluorescent in-situ hybridization (FISH), microarrays and next-generation sequencing (NGS) can also be employed on archival tissues, thereby playing a unique role in translational research, including biomarkers evaluation [9]. However, there are associated limitations that must be considered when utilising archival tissues, especially when they are fixed in the formalin for a long duration, or FFPE blocks are stored for a very long time. Prolonged fixation in the formalin and long-duration storing of archival tissues would affect the integrity of the DNA/RNA in these tissues [4]. Furthermore, exposing tissues to formaldehyde for a long duration can cause cross-linking of DNA protein. Additionally, the presence of paraffin as a leftover from sections of the FFPE block would inhibit the polymerase chain reaction amplification [8].

Variables like antigen retrieval method, antibody concentration, pH, temperature, detection method and incubation period have evolved throughout the optimisation process to produce the best possible environment for optimum antibody and antigen reactivity [10, 11]. Therefore, it is necessary to determine the optimal staining conditions for a specific antibody's reaction. A significant stage in optimisation is selecting the primary antibody concentration for specific staining while considering the volume of the diluent, temperature and

incubation duration [12]. In addition, antigen retrieval is essential to unmask the antibody-binding epitope, especially when tissues are fixed in the formaldehyde-based fixative. Another primary step in optimisation is to block non-specific antibody binding with hydrogen peroxide, sera or a protein to reduce background staining and potentially false-positive results [13].

The selection of a secondary antibody is crucial when employing an indirect detection method in IHC to optimise the visualisation of the primary antibody's distribution (Figure 1) [14]. Protein linker of either mouse or rabbit linker is a necessary condition that improves signal amplification of the primary antibody as it brings out a coloured reaction, which indicates an antigen of interest (Figure 1) [13, 15]. Retrieving the antigen epitope is an initial step in the IHC protocol, which could be achieved by using either heat-induced or proteolytic-induced methods. The exposed antigen will then react with the appropriate primary antibody, followed by the addition of secondary antibodies. An antibody linker could be included in some circumstances. To produce a coloured brown precipitate or product, as shown in Figure 1, a high sensitivity streptavidin- horse radish peroxidase (HSS-HRP) should be added subsequently, followed by the addition of diaminobenzidine (DAB), serving as chromogen for the development of final coloured precipitate [16, 17]

This study focuses on determining the expression of mismatch repair (MMR) proteins, i.e. MLH 1, MSH 2, MSH 6 and PMS 2 and their associated proteins of FOXP3 and BRAFV600E (Table 1). These markers are common and significant for colorectal cancer diagnosis in the clinical setting [18-20]. The MMR proteins are nuclear enzymes that help to correct base–base mismatches during DNA replication [21-23]. In addition, the proteins are associated with BRAFV600E mutations, and the associations are crucial in colorectal cancer development [24-29].

Table 1 List of IHC protein names and abbreviations

Name of protein	Abbreviation
Forkhead box P3	FOXP3
BRAF (mutated V600E)	BRAFV600E
MutL Protein Homolog 1	MLH1
MutS Protein Homolog 2	MSH 2
MutS Protein Homolog 6	MSH 6
Postmeiotic Segregation 2	PMS 2

Our IHC study involved a set of antibodies that were purchased from common antibody manufacturers of Abcam and Dako. The optimisation of the IHC procedures of each antibody was performed to ensure good quality of staining signals before it could be fully applied to the pathological tissues. In this report, we described how manipulating several technical features of IHC labelling could enhance the IHC signals of FOXP3, BRAFV600E and MMR antibodies.

2.0 METHODOLOGY

The IHC staining for FOXP3, BRAFV600E, MLH 1, MSH 2, MSH 6 and PMS 2 proteins was demonstrated by using a set of antibodies as described in Table 2.

Table 2 List of antibodies' names and abbreviations

Antibodies	Reactivity	Manufacturer
Mouse monoclonal Anti-FOXP3 (Ab20034)	Human	Abcam
Mouse monoclonal Anti-BRAF V600E (Ab22846)	Human	Abcam
Mouse monoclonal Anti-MLH1(M3640)	Human	Dako
Mouse monoclonal to Anti-MSH2 (M3639)	Human	Dako
Rabbit monoclonal Anti-MSH6 (M3646)	Human	Dako
Rabbit monoclonal to Anti-PMS2 (M3647)	Human	Dako

The FFPE blocks from the tonsil, brain and appendix tissues were cut at 5 µm and processed for IHC staining. Each tissue section was mounted on a Poly-L-Lysine microslide, dried on a hot plate and dewaxed in 2 changes of xylene. Afterwards, the tissues were hydrated by using decreasing concentrations of alcohol and rinsed in water. The tissues were pre-treated with Envision Flex Retrieval Solution, pH 9 (Dako), for the retrieval of the antigen epitope. This process was performed at 97°C for 20 minutes in Dako PT Link. Following that, the solution was left to cool down to 65^0C before it was brought out of the Dako PT Link. Then, the solution had to further cool down to room temperature. Each slide was transferred into a Tris Buffer Solution (TBS) (pH 7.6) filled to the brim to cover the entire tissue section in a carbon plane container and inserted into a Shandon Sequenza for manual IHC staining.

Following the recommended protocols, different primary antibodies were applied to other human positive control tissues (Table 3).

Table 3 Description of manufacturer's IHC steps for each antibody

Antibodies	**Positive controls**	**Cellular localisation**	**Antibody dilution**
Anti-FOXP3	Human mammary gland, human tonsil, and thymus tissue	Nucleus	1:500

Antibodies	Positive controls	Cellular localisation	Antibody dilution
Anti-BRAFV600E	Human melanoma tissue and brain tissue	Nucleus and cytoplasm	1:100
Anti- MLH1	Normal appendix tissue	Nucleus	1:50
Anti-MSH2	Normal appendix tissue	Nucleus	1:50
Anti-MSH6	Normal appendix tissue	Nucleus	1:50
Anti-PMS2	Normal appendix tissue	Nucleus	1:40

The dilution of each primary antibody was tested in a range of different dilutions following the recommended dilution by the manufacturer (Table 3). First, the prepared primary antibody dilutions were added to the slides at 100µL each, and the mixture was incubated for 1 hour. Next, the slides were washed with TBS buffer, and Envision Flex hydrogen peroxide (Dako, Germany) was added to each slide for blocking at 200µL and incubated for 5 minutes. Then, the envision Flex monoclonal mouse or rabbit linker at 150 µL was added to the slides that required a linker and allowed to act for 15 minutes. Subsequently, the horseradish peroxidase (HRP) from Dako (Germany) at 200 µL was added to the slides, followed by incubation for 20 minutes. The slides were then covered with DAB chromogen Dako (Germany) and allowed to act for 5 minutes. Following that, the slides were washed with distilled water [30-32].

The tissue sections were counterstained with Harris Haematoxylin solution for 10 seconds and rinsed with distilled water. They were dehydrated by increasing concentrations of alcohols starting from 70%, 80%, 95% and 2 changes of 100%. Afterwards, they were cleared in 2 changes of xylene and mounted with a coverslip using cytoseal mountant. To reduce bias during histopathologic evaluations, a blind assessment technique was employed [33]. Using this approach, reviewers examined the tissue samples without any prior knowledge of the identity of the sample. The first author and two pathologists who served as expert reviewers carried out the independent works. During a subsequent slide-viewing session, the pathologists jointly viewed the IHC slides using an Olympus multi-viewing microscope. The session was performed to get a consensus interpretation by comparing each case diagnosis to that of the first author [34]. Using x10 and x40 objective lenses, the IHC staining intensity, the proportion of positively stained cells, and the background staining clarity were observed [35]. All IHC images were taken using an Olympus XC-50 camera at x200 magnification.

3.0 RESULTS AND DISCUSSION

Blue arrows indicate the positive FOXP3 at nuclear staining in Figure 2. Dako Envision Flex monoclonal mouse linker was added to enhance the intensity of FOXP3 concerning different primary antibody dilutions of 1:50, 1:100, 1:200, 1:300 and 1:500 and incubated for 15 minutes. We discovered that the lower concentration of primary antibody at dilution of 1:300 produced a comparable intensity of FOXP3 nuclear than the higher concentration of primary antibody at dilution 1:50 (Figure 2). FOX3P3 positive staining was previously demonstrated by using a lower primary antibody dilution of 1:500 [19] and 1:600 [33]. The findings of this study, however, suggested that the FOXP3 positively stained at 1:500 dilution was less intense when compared to the 1:300 dilution (Figure 2). The optimisation procedure also found that the 1:50 dilution of FOXP3 produced an extremely intense, noisy background. However, the staining background decreased as the primary antibody of FOXP3 was diluted (Figure 2). With the above matters, the primary antibody incubation at 1:300 was selected as the optimal dilution for IHC staining of FOXP3.

BRAFV600E-positive nuclear and cytoplasmic staining was reported by Capper *et al.* [2], Saizul *et al.* [11], Luey *et al.* [29], Loes *et al*. [34] and Lanza *et al.* [36], which was confirmed in our study using positive control normal brain tissues (Figure 3). The recommended antibody concentration of BRAFV600E was indicated at the dilution of 1:100 (Table 3) [37]. The incubation of BRAFV600E primary antibody at a lower concentration of a dilution of 1:200 resulted in a decreasing staining intensity than the recommended dilution (Figure 3). Furthermore, the tissues were incubated at 1:100 dilutions with a linker treated overnight, resulting in substantial BRAF nuclear and cytoplasmic staining (blue arrows, Figure 3). A comparable BRAFV600E dilution at 1:100 was also demonstrated by earlier studies [38–41]. Contrary to our findings, Roseweir *et al.* reported that the optimal BRAFV600E dilution was 1:200 dilution [42]. The investigation's use of citrate buffer as the antigen retrieval reagent, 10% casein as a blocking solution, and 3% hydrogen peroxidase to inhibit endogenous peroxidase activity highlighted the different conditions used in their setting compared to ours. In addition, in contrast to our study, the report [42] also detailed the use of histoclear as a dewaxing reagent. The reagent was demonstrated as an improved histology result-generating replacement for xylene [42, 43].

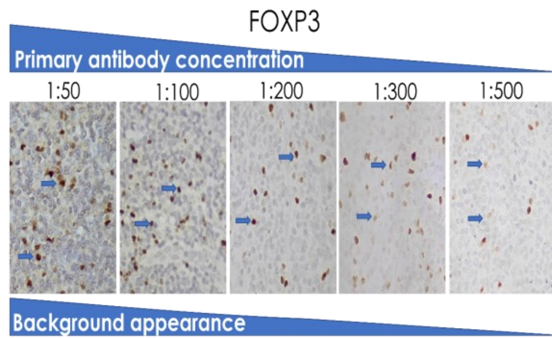

Figure 2 IHC staining for FOXP3 in positive control normal tonsil tissues, x200 magnification

However, we observed background staining in BRAFV600E-optimised slides with a linker even at a lower concentration of 1:200. This might be due to the brain tissue composition reported in the previous studies [42-44]. Masuda *et al*. (2021) recommended a 6-15 µm when handling the neuropathology tissues in IHC [35]. This is due to the cut of the brain tissues, which are slightly thicker than other tissues; therefore, they would absorb more of the primary antibody and increase the background staining. However, this did not affect the assessment of the BRAFV600E-positive cells, as indicated in Figure 3. Hua *et al*. [46] reported that it is still challenging to consistently generate high contrast staining over thick sections of tissue samples in neuronal tissue.

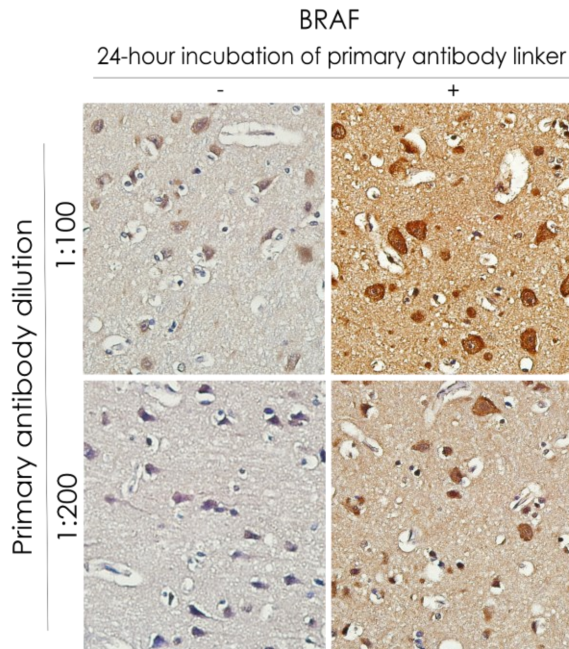

Figure 3 IHC staining for BRAFV600E in positive control normal brain tissues, x200 magnification

IHC staining was optimised to a panel set of MMR antibodies (Figure 4). As indicated by the blue arrows, the positive staining of MLH1, MSH2, MSH6 and PMS2 was determined at nuclear and cytoplasmic localisation (Figure 4). Through positive control of normal appendix tissues, it was confirmed that the manufacturer's best optimal dilutions for MLH1, MSH2 and PMS2 antibodies followed the recommended primary antibody concentration at a dilution of 1:50 (Table 3). A similar technique for the IHC staining for MLH1 and MSH2 was also performed in previous studies [31, 32]. Figure 4 demonstrates that incubating primary antibodies at a lower concentration decreases the staining intensity of MLH1, MSH2 and PMS2. However, the incubation of the primary MSH6 antibody at a dilution of 1:50 produced a more non-specific staining background (Figure 4). These non-specific backgrounds were reduced as tissues were incubated at a lower primary antibody concentration.

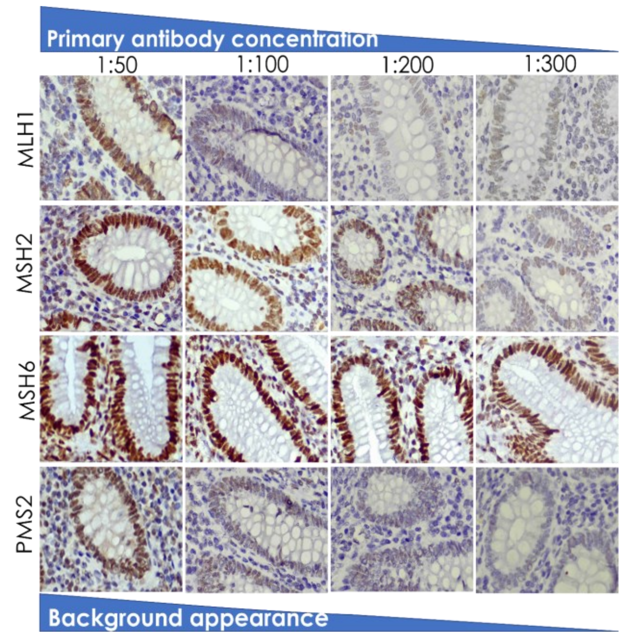

Figure 4 IHC staining for MMR proteins; MLH1, MSH2, MSH6 and PMS2 in positive control normal appendix tissues. Primary antibodies of MSH2 and MSH6 are incubated for 1 hour while MLH1 and PMS2 are incubated overnight, x200 magnification

Nonetheless, the incubation of primary antibody at the dilution of 1:50 was considered for the higher concentration [31, 32]. As a result, IHC staining using an increased concentration might consume higher primary antibody volume that can only be applied for minimal sample reactions. Furthermore, the use of excessively high antibody concentrations that result in background staining and non-specific reactions is one of the most frequent causes of false-positive signals in IHC preparation [32]. To overcome this limitation, the incubation of primary antibody steps was optimised by incorporating an overnight incubation of the antibody linker in a lower primary antibody concentration at the dilutions of 1:100 and 1:200.

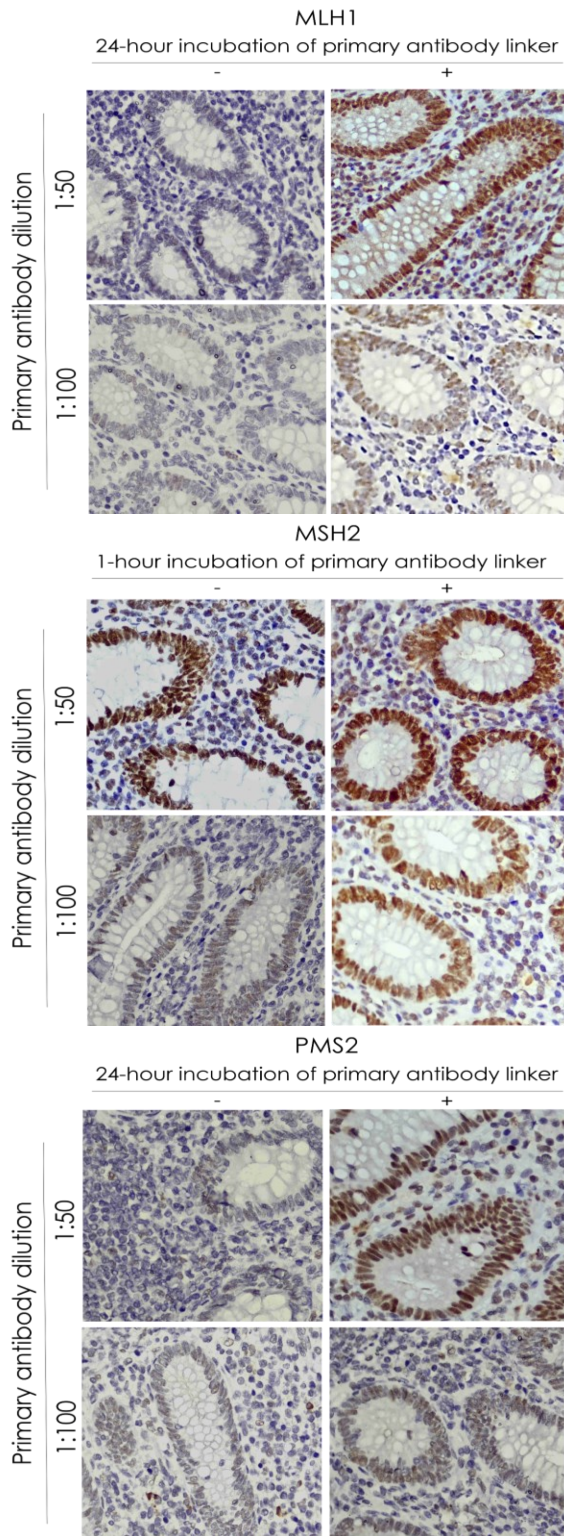

Figure 5 IHC staining for MLH1, MSH2 and PMS2 in positive control normal appendix tissues, x200 magnification

In Figure 5, we tested whether the primary antibody linker has a role in increasing the intensity of MLH1 and PMS2 in normal appendix tissues incubated with primary dilutions of 1:50 and 1:100 overnight. From the figure, the staining intensity of dilutions at 1:100 with antibody linker did not enhance the staining intensity of MLH1 and PMS2. The results suggested that the addition of a primary antibody linker for overnight incubation at 1:100 dilution did not improve the reactivity of the MLH1 and PMS2 monoclonal antibody at a lower concentration. Therefore, the optimal concentration of MLH1 and PMS2 was suggested at a dilution of 1:50 overnight, which follows the recommended antibody dilution by a manufacturer (Table 3)

The dilution of the primary antibody incubation was tested at 1:50 and 1:100 with a linker for MSH 2. The positive MSH 2 at nuclear staining was indicated by the blue arrows. To enhance the intensity of the IHC staining, an hour of incubation of the Dako Envision Flex monoclonal rabbit linker was performed. The left panel shows the positive control MSH 2 tissues that have not been treated with the primary antibody linker. In contrast, the right panel shows tissues that have been incubated for an hour with the primary antibody linker. All pictures were taken under a light microscope with x 200 magnification.

Figure 6 demonstrated the results of MSH6 nuclear staining after 1-hour incubation of primary antibody with and without the primary antibody linker. The enhancement of MSH6 staining intensity was seen in tissues incubated with a lower primary antibody concentration at dilutions of 1: 100 and 1:200 (Figure 6). Due to the staining intensity enhancement and the minimal appearance of a non-specific staining background, the concentration of MSH6 at a dilution of 1: 100 with the incubation of the primary linker was selected as the optimal staining step for the MSH6.

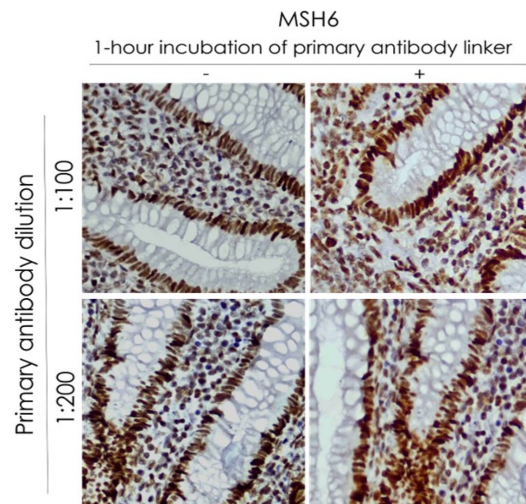

Figure 6 IHC staining for MSH6 in positive control normal appendix tissues, x200 magnification

The summary of the optimised IHC staining conditions for FOXP3, BRAFV600E and MMR antibodies is shown in Table 4. Even though one specific dilution is recommended by the antibody's manufacturer (Table 3), the optimisation staining is still

needed to determine the ideal IHC results according to the lab settings.

Table 4 Summary of the optimized IHC staining for each antibody

Antibodies	*Selected primary ab dilution	Incubation time, temperature	The appearance of staining background
Anti-FOXP3	1:300	1hr, RT	Not available
Anti-BRAFV600E	1:100	Overnight, 4°C	Available
Anti- MLH1	1:50	Overnight, 4°C	Not available
Anti-MSH2	1:50	1hr, RT	Not available
Anti-MSH6	1:100	1hr, RT	Not available
Anti-PMS2	1:50	Overnight, 4°C	Not available

**The additional primary antibody linker was applied in all primary antibodies.*

A significant stage in IHC optimisation is choosing the suitable primary antibody with positive cross-reactivity of the target tissue's antigen [42, 43]. Additionally, to ensure the positivity of the antibody's staining, the primary antibody concentration and the incubation time with the right temperature should be considered [44-46]. Our optimisation study determined that the FOXP3 and MSH6 worked well at a lower concentration of 1: 300 and 1:100, respectively. Meanwhile, the MLH1, MSH2 and PMS2 required a higher concentration of dilution at 1:50. Heat antigen retrieval using Envision Flex retrieval solution was shown to produce positive IHC staining for all antibodies used in this study. In addition, tissues treated with the Envision Flex Hydrogen Peroxide (Dako, Germany) appeared to have minimal non-specific antibody binding.

The optimisation step done included Dako HRP detection system that produced positive signalling in all antibodies from the same manufacturer and the Abcam manufacturer. There are 2 types of staining in IHC which are direct staining, which involves labelled primary antibody and indirect staining method, which uses labelled secondary antibody against the primary antibody [47-49]. Both direct and indirect IHC staining can be detected using visible light detection, fluorescent detection or electron detection, depending on the type of labelled chromogen [13, 44]. A secondary antibody labelled with biotin or fluorescent dye (for example, FITC and Alexa-fluor) or a chromogenic enzymatic tag (for example, HRP and alkaline phosphatase) is typically used to detect the primary antibody [48]. An HRP-conjugated secondary antibody directed towards the primary antibody is generally employed as it is more specific, sensitive and versatile than a labelled primary antibody only [50-52]. Substrates such as 3,3'-diaminobenzidine (DAB) and 3-amino-9-ethylcarbazole (AEC) are used to visualise HRP-conjugated staining to detect the presence or absence of the targeted antigen [53-55]. Chromogenic detection can detect the presence of an antigen when an enzyme transforms a soluble substrate into an insoluble coloured product that is deposited at the site of antigen expression. The detection is achieved by DAB and AEC into brown and red end products, respectively. The enzymes horseradish peroxidase (HRP) and alkaline phosphatase perform the chromogenic detection process [14, 56, 57]. DAB is more often utilised than AEC due to its durability and resistance to fading when exposed to light.

In chromogenic detection, the signal is amplified by using the Labeled-Avidin-Biotin-Complex (LABC) and Labeled-Streptavidin-Biotin (LSAB). The avidin-biotin-peroxidase combination enabled the LABC to perform its function [13, 14]. In contrast, the LSAB links the labelled secondary antibody to the primary antibody that is bound to the target antigen using the streptavidin-peroxidase complex [57-59]. Compared to LABC, which produces bigger complexes, LSAB has the advantage of forming smaller complexes that can easily permeate the tissue, improving its sensitivity. Endogenous biotin negatively impacts both LABC and LSAB. Due to this, the biotin must be blocked during IHC staining to prevent the tissues from becoming more stained in the background. In recent years, antibodies and enzymes have been coupled to the polymer backbone, which provides more sensitivity and specificity than LABC and LSAB [13, 57, 60].

The use of a primary antibody linker has been demonstrated as an essential component in the current IHC principles (Figure 1). This protein linker has been discussed to improve the IHC signal of the primary antibody [13, 15]. Our study showed that the Envision Flex monoclonal mouse or rabbit linker (DAKO) improved the IHC signals for FOXP3, BRAFV600E and MSH6 at lower primary antibody concentrations. However, the inclusion of a primary antibody linker was not seen to enhance the intensity of MLH1, MSH2 and PMS2 IHC signals.

4.0 CONCLUSION

In conclusion, when using archival tissues, modifying the IHC staining variables such as antigen retrieval conditions i.e. pH and temperature, primary antibody concentration and incubation period improves staining for the MMR, BRAFV600E, and FOXP3 proteins. Unfortunately, the different options for the aforementioned IHC staining variables appear to be limitless. A comparison of the two antigen retrieval methods, heat-induced epitope retrieval (HIER) and proteolytic-induced epitope retrieval (PIER), choosing the appropriate blocking solution and antibody diluent, and secondary antibody for the IHC

detection signal are additional aspects of IHC optimization that are not covered in our study. Researchers frequently employ TBS as a diluent, however the choice of diluent can significantly affect how well an antibody performs. Similarly, the detecting technique used might have a considerable influence on staining quality. Even though HRP secondary antibodies generate an improved signal and clearer background, more concentration modification is probably required to improve the IHC outcomes. Above all, even the effort took a few weeks to validate antibody specificity and enhance the staining process, we learned that our optimisation work was certainly worthwhile for our study and to direct future IHC research pertaining to the relevant antibodies.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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