MATERIALS AND METHODS

CHAPTER THREE: MATERIALS AND METHODS

3.0 STOCK SOLUTIONS

The composition and procedure for preparation of all stock solutions used in the study are described in the appendix.

3.1 BIOPSY MATERIAL

Formalin-fixed, paraffin-embedded archival tissue were used as samples for this study. These were obtained from the Seremban General Hospital, Negeri Sembilan and the Dental Faculty of University Malaya.

The histopathological status of these tissues were established based on Hematoxylin and Eosin stained sections obtained from the paraffin block at the start and the end of sections used in the study.

Four micron-thick sections were sliced and placed onto 5 pre-treated 2-well teflon-coated slides for each sample. Preparation of pre-treated slides is described in section 3.7. Five consecutive sections were placed into sterile 1.5 ml microfuge tubes (Eppendorf). A new disposable blade was used for each sample block to prevent contamination between samples.

3.2 METHODS OF DNA EXTRACTION

3.2.1 Extraction of DNA from paraffin-embedded tissue

Five 4 micron-thick sections of paraffin-embedded tissue were placed into a sterile 1.5 ml microfuge tube. The sections were heated at 56°C for 15 minutes and deparaffinized with 1 ml of xylene by inverting the tube gently for 15 minutes at room temperature. The tube was centrifuged in a microfuge (Sorvall Instruments) at 13000 rpm for 5 minutes. The xylene was carefully removed with a sterile plugged Pasteur pipette so as not to damage the tissue. The pellet was resuspended in xylene and the procedure repeated.

Residual xylene was removed by adding 0.5 ml of 100% ethanol. The tube was inverted to mix the contents. The mixture was centrifuged at 13000 rpm for 5 minutes and the ethanol removed. The pellet was resuspended in ethanol and the procedure repeated. The pellet was dried in a speed vacuum concentrator (Savant) and resuspended in 50µl of sterile double distilled water. The tube was then frozen at -70°C for 45 minutes.

The sample was thawed at room temperature and 50 µl of proteinase K mix was added. The mixture was incubated in a 55°C water-bath for one hour and centrifuged at 12000 rpm, 4°C for 15 minutes in a

microfuge (Sigma Co.). The supernatant was stored at 4°C and used as template for PCR assays.

3.2.2 Extraction of DNA from throat washes

Throat washes were obtained from healthy volunteers known to continuously shed HHV-6 in saliva. Viral DNA was extracted and used as a positive control for PCR assays along with other HHV-6 DNA positive controls. The throat wash was centrifuged at 20000 rpm for 1 hour at 4°C in a centrifuge (Beckman, J2-M1, Rotor 20). The supernatant was discarded and the pellet resuspended in 200 µl of digestion buffer containing Proteinase K at a final concentration of 200 µg/ml. The suspension was incubated at 37°C overnight, RNase was added to the suspension and incubated at 37°C again for 2 hours.

DNA was extracted by phenol-chloroform treatment and ethanol precipitation. The DNA pellet was resuspended in 40 µl of 1X PCR buffer (Perkin-Elmer Cetus Co.) and stored at 4°C. Ten microlitres was used in PCR experiments.

3.2.3 Plasmid isolation

Plasmid pZVH14 was transformed into supercompetent JM101 *E.coli* cells and propagated in Luria Bertani (LB) broth using 50 µg/ml of Ampicillin as a selective antibiotic. A single colony of bacteria was inoculated into 3 ml of LB broth and Ampicillin mixture and incubated at 37°C. At log phase, the bacterial mixture was transferred into 97 ml of LB broth with Ampicillin, and incubated overnight in a controlled environment incubator shaker at 37°C. Plasmid DNA was extracted by alkali-lysis as specified by Sambrook and colleagues (1989).

The bacterial culture was split into two 50 ml Oakrigde tubes (Nalgene) and centrifuged at 4000 rpm, 4°C for 15 minutes. The supernatant was discarded and the pellet resuspended in 2 ml of Solution I. Four millilitres of freshly prepared Solution II was added and the contents mixed thoroughly by inverting the tube several times. The mixture was incubated at room temperature for 5 minutes. Three millilitres of ice-cold Solution III was added, the contents mixed several times and stored on ice for 10 minutes.

The bacterial lysate was centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant carefully transferred into a new centrifuge tube. DNA was precipitated with 0.6 volume of isopropanol and spun down at 5000 rpm for 15 minutes at room temperature. The

supernatant was discarded and the DNA pellet was washed with 70% ethanol, dried in a speed vacuum concentrator and resuspended in 500 µl of 1X PCR buffer.

Five microlitres was used as a positive control for PCR assays. Subsequently, the plasmid was also labelled and used in Southern hybridization.

3.3 POLYMERASE CHAIN REACTION

PCR amplification of DNA was carried out with the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus) according to the methods described by Saiki *et al.* (1985) and Mullis and Faloona (1987).

Sterile, 0.5 ml siliconized microcentrifuge tubes (Perkin-Elmer Cetus), yellow tips, white tips and double distilled water were placed in a Laminar flow chamber (Gelaire Flow Laboratories) and kept sterile under ultraviolet (UV) light. Aliquotting of reagents was carried out in the chamber, which was only used for PCR experiments. Reagents were aliquotted into the microcentrifuge tubes using positive displacement pipettors (Eppendorf). A PCR cocktail was prepared as shown in Table 1. The volume of template used was determined by quantification of samples.

This cocktail was overlaid with 40 µl of light mineral oil (Sigma Co.) to prevent evaporation during amplification. The tubes with the PCR cocktails were then placed in the DNA Thermal Cycler (Perkin-Elmer Cetus) and programmed specifically for each PCR assay.

Table 3.1: PCR cocktail for the amplification of primer-directed DNA sequences

Reagent	Volume (μl)	Final concentration
Sterile double distilled water	variable	
PCR buffer (with $MgCl_2$ and gelatin) 10X concentration	5	1X
dNTP mix (dATP, dCTP, dGTP, dTTP)	1	200μM each
Primer 1	0.5	20pmole
Primer 2	0.5	20pmole
Taq DNA polymerase	0.5	2.5U
DNA template	variable	100ng
Final volume	**50**	

3.3.1 Primers and controls used in PCR assays

We used three sets of primers which were synthesized by Genosys Biotechnologies, Inc. (USA) (Table 2). The first set of primers amplified a β-globin gene sequence. The other two sets of primers were used in a nested protocol that amplified specific sequences of HHV-6 large tegument protein.

All PCR assays included the use of positive controls to ensure the reliability of each run and the amplification of the positive control signified that the run was a success. Various DNA controls, namely saliva DNA positive for HHV-6, plasmid pZVH14, DNA from U1102 strain propagated in HSB-2 cell line (Advanced Biotechnologies Inc., USA) and DNA from Z-29 strain propagated in human cord blood lymphocytes (Advanced Biotechnologies Inc., USA) were used.

Negative controls were included to eliminate the possibility of product carryover. Extracts from JM101 *E.coli* cells without the pZVH14 insert, empty paraffin block sections and sterile double distilled water were used as negative controls in PCR experiments.

Ten microlitres of supernatant from extracted paraffin-embedded tissue samples were subjected to PCR amplification of a β-globin gene sequence with primers PC04 and GH20. This procedure was

carried out to ensure that DNA was present and amplifiable within each extracted sample.

3.3.2 PCR amplification of HHV-6 DNA sequences

Subsequently 10 µl of extracted sample tissues was used for first round PCR amplification with the external primers A and C. These synthetic primers, which were derived from the SIE strain nucleotide sequence by Collandre and colleagues (1991), amplified an 830 basepair DNA segment in the putative LTP gene of HHV-6. All PCR products from the first round of amplification was subjected to a 'nested' protocol to further increase the possibility of viral DNA detection. One microlitre of each PCR product was reamplified with internal primers HS6AE and HS6AF. These primers amplified an approximately 750 basepair fragment within the 830 basepair region. The amplification parameters for first and second round PCR cycles are shown in Table 3.3.

Table 3.2: Primers used in PCR assays

Set	Primer	Sequence (5'→3')	Size	Reference
I	β-globin		268 bp	Perkin Elmer
	PC04	CAACTTCATCCACGTTCACC		Cetus Co.
	GH20	GAAGAGCCAAGGACAGGTAC		
II	HHV-6		830 bp	Collandre *et al.*,
	A	GATCCGACGCCTACAAACAC		1991;
	C	CGGTGTCACACAGCATGAACTCT C		Aubin *et al.*, 1991
III	HHV-6		750 bp	Dewhurst *et al.*,
	HS6AE	CGGCCATTTAACGGAACCCTAG		1993
	HS6AF	TCCAGAGAAAGGGTGTTGCG		

Table 3.3: Parameters for the amplification of HHV-6 DNA sequences

Stage	Denaturation at 92°C (min)	Annealing at 60°C (min)	Extension at 72°C (min)	Cycles
I	7	1	1.25	1
II	1	1	1	38
III	1.25	1.25	7	1

3.4 AGAROSE GEL ELECTROPHORESIS

All PCR products were electrophoresed on 2% agarose gels (Sigma Chemicals Co.) prepared with 0.5X TBE. Eight microlitres of amplified PCR product was added to gel loading dye and dispensed into the respective wells with a micropipettor. A 100 basepair molecular weight marker (Gibco, Bethesda Research Lab., U.S.A) was included in each run. Electrophoresis was carried out with 0.5X TBE as the electrophoresis buffer at a constant voltage of 120V for approximately 3 hours.

After ethidium bromide staining (final concentration of 0.5μg/ml), the gel was destained with distilled water and visualized over a long wavelength UV transilluminator (Model TS 36, UV products, Inc.). Results were documented on Polaroid 665 film with a Polaroid MP-4 land camera.

3.5 NONRADIOACTIVE SOUTHERN BLOTTING

The Southern Blot technique using the Nonradioactive DNA Labelling and Detection Kit was carried out as described by Boehringer Mannheim (1993).

3.5.1 Nonradioactive probe labelling

The plasmid pZVH14 was PCR-labelled with the Dig DNA labelling mixture (10X concentration) from the Nonradioactive DNA Labelling and Detection Kit.

Two microlitres of amplified pZVH14 was used as template in the labelling experiment which involved PCR labelling and amplification using Dig DNA labelling mix instead of the dNTP mix. The PCR cocktail specified in Table 3.4 was amplified with the parameters as shown in Table 3.3.

The efficiency of the labelling reaction was determined by estimating the yield of Dig-labelled probe as described by Boehringer Mannheim (1993). The probe was diluted with sterile distilled water to give a final concentration of 10 fmol/µl. Eight microlitres of the diluted probe was used in hybridization experiments.

Table 3.4: Reaction mix for the PCR labelling of probe sequences

Reagent	**Volume (µl)**	**Final concentration**
Sterile double distilled water	39.5	
PCR buffer (with $MgCl_2$ and gelatin) 10X concentration	5	1X
Dig DNA labelling mixture 10X concentration	2	0.4X
Primer HS6AE	0.5	20pmole
Primer HS6AF	0.5	20pmole
Taq DNA polymerase	0.5	2.5 U
DNA template (pZVH14)	2	100ng
Final volume	**50**	

3.5.2 Southern Transfer

The Southern transfer of DNA from agarose gels to nylon membranes was a modification of the method described by Maniatis and colleagues (1982).

The agarose gel was transferred to a glass baking dish and soaked in 100 ml of denaturation buffer to denature the DNA. The gel was then neutralized in 100 ml of neutralization buffer. Both steps were carried out for 30 minutes at room temperature with constant shaking.

A two centimetre-thick plexiglass platform, larger than the gel was wrapped with Whatman 3MM paper and placed inside a large baking dish. The dish was filled with 10X SSC just below the top level of the platform. The gel was placed on the platform with the wells facing downwards. Air bubbles trapped between the gel and the platform were smoothed out by rolling a clean glass pipette over them.

A nylon membrane (Hybond N^+) and 2 pieces of Whatman 3MM paper were cut to the size of the gel and wet in 2X SSC. The nylon membrane was placed on the gel, followed by the Whatman 3MM paper. Trapped air bubbles were smoothed out with the pipette.

An eight centimetre-high stack of absorbent paper towels, cut to the size of the gel, was placed on the 3MM paper. A paper weight of 500 gm was placed on the stack and transfer of DNA from the gel to the

membrane was carried out for 24 hours by capillary flow. As an added precaution, the gel was surrounded by a water-tight border of Saran Wrap to prevent short-circuiting of fluid between the paper towels and the 3MM paper under the gel.

The dehydrated gel was restained and viewed under UV illumination to ensure complete transfer of DNA. The membrane was soaked in 6X SSC for 5 minutes, dried on 3MM paper and UV cross-linked for 3 minutes to fix the DNA onto the membrane.

3.5.3 Nonradioactive hybridization

The membrane was placed between 2 cloth meshes, wet in 2X SSC and rolled into a neat cylindrical shape. It was placed into a hybridization cylinder (Hybaid) and prehybridized with 20 ml of hybridization solution in a hybridization oven (Hybaid) at 68°C for 1 hour.

The prehybridization solution was discarded and replaced with 3 ml of hybridization solution containing 8 µl of labelled DNA probe, freshly denatured at 94°C for 6 minutes. Hybridization was carried out at 68°C for 16 hours.

The membrane was removed and subjected to low stringency washes twice with Wash Solution 1 for 10 minutes at room temperature each time. High stringency washes were carried out twice with Wash

Solution 2 at 68°C for 30 minutes each time. Both stringency washes involved constant shaking.

3.5.4 Detection of hybridized products

The following incubations were carried out at room temperature and required constant shaking, with the exception of the colour reaction.

The membrane was washed briefly in buffer 1, incubated in buffer 2 for 30 minutes and washed in buffer 1 again. The membrane was then incubated with 150 mU/ml of Anti-digoxigenin-Alkaline phosphatase in buffer 1 for 30 minutes. The unbound antibody-conjugate was removed by washing twice with buffer 1 for 15 minutes each time. The membrane was then equilibrated with 20 ml of buffer 3 for 2 minutes.

The membrane was then sealed in a plastic bag containing freshly prepared colour solution and incubated in the dark. The reaction was monitored until the desired bands were detected. The membrane was then washed with buffer 4 for 5 minutes to stop the reaction and the results were documented.

3.6 RESTRICTION ENDONUCLEASE CHARACTERIZATION

PCR products that amplified HHV-6 DNA sequences were subjected to digestion by the enzyme *Hind* III (New England Biolabs, Inc.) with the recognition site for 5' A↓AGCTT 3'. All the components were added into a sterile microcentrifuge tube as shown in Table 3.5.

Table 3.5: Reaction mix for restriction endonuclease digestion

Reagent	**Volume (µl)**
Sterile, double distilled water	8
NE Buffer 2 (10X concentration)	2
Amplified DNA	8
Hind III	2
Total volume	**20**

The reaction mixture was incubated at 37°C for 18 hours. The digested product was analyzed by agarose gel electrophoresis and the results documented on Polaroid 665 film.

3.7 PREPARATION OF SLIDES

Pre-treated slides were prepared for tissue sections using a modification of the method described by Boehringer Mannheim (1989). All washes and incubations were carried out at room temperature unless stated otherwise.

Two-well teflon-coated slides were boiled in 0.1 M HCl for 5 minutes. When cool, the slides were removed and washed twice in distilled water for 5 minutes each time. The slides were incubated in 100% ethanol for 30 minutes, air-dried and incubated in 1X Denhardt's solution overnight.

The slides were fixed in a mixture of alcohol : glacial acetic acid at 3 : 1 ratio for 20 minutes. Decreasing concentrations of alcohol, 100%, 70% and 50% were used to hydrate the slides by soaking for 5 minutes each time. This was followed by an overnight incubation in 1% gamma-aminopropyltrithoxysilane solution (pH 3.45) at 70°C in a sterilizing oven (Memmert SLM 600).

The slides were washed five times in distilled water for 5 minutes each time and dried overnight in the oven at 80°C. These treated slides could be stored for a period of 6 months prior to activation.

Activation of slides was carried out in a fume chamber with 10% (v/v) glutardialdehyde in phosphate-buffered saline (PBS) pH 7.6 for 30 minutes. The slides were washed twice in distilled water for 5 minutes each. Stabilization with 0.1M sodium-m-periodate was also carried out in the fume chamber for 15 minutes.

The slides were washed in PBS three times for 5 minutes each time and dried overnight in the oven at 42°C. Paraffin-embedded tissue sections were then fixed on to these slides.

3.8 NONRADIOACTIVE *IN SITU* HYBRIDIZATION

All washes and incubations were carried out at room temperature unless stated otherwise. Phosphate buffered saline (PBS) was used as a rinsing buffer at pH 7.2.

3.8.1 Nonradioactive oligonucleotide probe labelling

HHV-6 variant specific oligonucleotide probes were synthesized by Genosys Biotechnologies, Inc. as described below (Dewhurst *et al.*, 1993).

HHV-6 A probe 5' - AAAACATTGAAGAAGTTT - 3'

HHV-6 B probe 5' - AAGACATTGAAGAAGCTT - 3'

Both probes were labelled by a standard oligonucleotide 3'-tailing reaction with Digoxigenin-11-dUTP/dATP from the Dig Oligonucleotide Tailing Kit (Boehringer Mannheim). All reagents were aliquotted into a sterile microfuge tube stored on ice in the following order as shown in Table 3.6.

The microfuge tube was incubated at 37°C for 15 minutes and placed on ice. One microlitre each of 20 mg/ml Glycogen solution and 200 mM EDTA (pH 8.0) was added to the tube. The labelled

oligonucleotide was then precipitated with 2.5 µl of 4M LiCl and 75 µl of pre-chilled absolute ethanol. The mixture was incubated at -70°C for 30 minutes.

The reaction tube was centrifuged at 13000 g for 15 minutes at 4°C. The supernatant was removed and the pellet washed with cold 70% ethanol. The tube was centrifuged again for 5 minutes and the ethanol removed carefully. The pellet was dried in the speed vacuum concentrator, resuspended in 20 µl of sterile double distilled water and stored at -20°C.

The labelling efficiency of both probes were determined as described by Boehringer Mannheim (1993). The labelled probes, A and B were diluted to give a final concentration of 100 ng/ml each.

Table 3.6: Reaction mix for 3'-tailing of oligonucleotide probes

Reagent	**Volume (µl)**	**Final concentration**
Reaction buffer (5X concentration)	4	1X
$CoCl_2$ solution	4	5mM
Dig-11-dUTP	1	0.05mM
Oligonucleotide	2	5µM
dATP	1	0.5mM
Terminal transferase	1	2.5U/µl
Sterile double distilled water	7	
Total volume	**20**	

3.8.2 Nonradioactive *in situ* hybridization

Each slide contained two paraffin-embedded tissue sections from one sample. These tissue sections were heated at 56°C for 15 minutes and deparaffinized by immersion in xylene for 3 minutes. The tissues were treated in decreasing concentrations of graded alcohol as follows, 100%, 95%, 90%, 80% and distilled water for 2 minutes each to hydrate the cells.

The tissues were incubated in 0.2N HCl for 20 minutes to digest the proteins. The slide was then washed in distilled water and PBS for 5 minutes each. Permeabilization of cell membrane was carried out with 100 μg/ml of Proteinase K at 37°C for 15 minutes. The slide was washed with 0.2% Glycine in PBS for 10 minutes.

The sections were post-fixed with freshly prepared 4% paraformaldehyde for 20 minutes and washed with PBS for 5 minutes. The slide was then washed with 1X SSC for 10 minutes and dehydrated in increasing concentrations of graded alcohol as follows: 80%, 90%, 95% and 100% for 1 minute each.

A probe cocktail was prepared as a stock solution for prehybridization and hybridization as shown in Table 3.7. Prehybridization solution consisted of all components except for the Dig-labelled probe. Prehybridization was carried out by incubating tissue sections with 50 μl of

prehybridization solution in a humidified chamber for 1 hour at room temperature.

Following a brief wash in 2X SSC, the area surrounding both wells of the slide was dried with a piece of Whatman 3MM paper, taking care not to dry the sample tissue. Each well was then circled with silicone glue (Loctite Co., USA). Fifty microlitres of the probe cocktail containing 100 ng/ml of digoxigenin-labelled probe was applied to each well, that is, probe A to the first well and probe B to the second well. The slide was then covered with siliconized cover slips (Hirschmann Lab., Germany).

Denaturation of cell and probe DNA was carried out by placing the slide on a heated plate in the Omnigene thermal cycler (Hybaid Ltd., U.K.) for 6 minutes at 95°C. The slide was immediately chilled on ice and then incubated in a humidified chamber at 42°C overnight.

Table 3.7: Probe cocktail for *in situ* hybridization

Reagent	Volume (μl)	Final concentration
50X Denhardt's solution	10	1X
50% (w/v) Dextran sulphate	50	5%
Sonicated salmon sperm DNA	10	0.2mg/ml
20X SSC	100	4X
Sterile double distilled water	variable	
100% deionized Formamide	250	50%
Dig-labelled probe	variable	100ng/ml
Final volume	**500**	

3.8.3 Detection of hybridized products

The silicone glue and coverslip were removed carefully. The slide was subjected to low stringency washing with 2X SSC and 1X SSC for 1 hour each time. A high stringency wash was applied at 42°C for 30 minutes with 0.5X SSC to effectively remove non-specific binding of probe to cellular DNA. The slide was then washed with 0.5X SSC for 30 minutes.

Bound probes were visualized by colour detection according to the Nonradioactive DNA Labelling and Detection Kit (Boehringer Mannheim). The slide was briefly washed in buffer 1 and incubated with buffer 2 for 30 minutes. Once again the slide was washed in buffer 1 and incubated with 150 mU/ml of Anti-digoxigenin-Alkaline phosphatase diluted in buffer 1 for 30 minutes.

Unbound antibody-conjugate was removed from the slide by washing with buffer 1 twice for 15 minutes each. The tissue was equilibrated in buffer 3 for 2 minutes. Each well was then incubated with 100 µl of freshly prepared colour solution and placed in a humidified chamber. The chamber was wrapped in aluminium foil and incubated in the dark for 18 hours. The reaction was stopped by washing the slide with buffer 4 for 5 minutes.

The tissue was counterstained with Mayer's hematoxylin (Fluka) for 2 minutes and rinsed in distilled water. The slide was then immersed in ammonia water for 10 seconds and rinsed in distilled water again. Slides were mounted with glycergel (Dako Co.) that was pre-heated at 50°C and left to dry in the dark. Results were visualized under a light microscope (Olympus, Japan).

3.8.4 Controls used in nonradioactive *in situ* hybridization

Every *in situ* hybridization run included the use of acetone-fixed MOLT-3 cells infected with HHV-6 variants A and B as a positive control to ensure the success of the experiment. Negative controls included the use of uninfected MOLT-3 cells and the omission of labelled probe during hybridization of HHV-6 infected MOLT-3 cells. Known negative sample tissues were also hybridized concurrently with other samples using the labelled probe to eliminate false positive signals due to background staining.

3.9 IMMUNOHISTOCHEMISTRY

Two monoclonal antibodies (MAb) to HHV-6 variants A and B were used in this study. MAb 9A5D12 (Advanced Biotechnologies Inc.) which reacts with HHV-6 p41/38 early antigen (Balachandran *et al.*, 1989) was diluted in PBS (pH 7.6) to give a final concentration of 12.5 μg/ml. MAb 6A5G3 (Virotech International Inc.) which reacts specifically with HHV-6 gp116/64/54 (Balachandran *et al.*, 1989) was used at a final concentration of 20 μg/ml of PBS. Both MAbs were highly specific to HHV-6 with no reactivity detectable to other herpesviruses (Advanced Biotechnologies Inc., Virotech International Inc.).

MOLT-3 cells infected with HHV-6 A and B were fixed on glass slides and used as positive controls in each immunohistochemical experiment. A negative control was also included by using uninfected MOLT-3 cells, omitting the MAb or by using a known negative paraffin-embedded tissue sample.

Immunohistochemical staining was carried out on formalin-fixed, paraffin-embedded tissue sections that were fixed on to activated slides processed as described in section 3.7. Tissue sections were heated at 56°C for 15 minutes and deparaffinized with xylene. The sections were

then hydrated in decreasing concentrations of graded alcohol as follows: 100%, 95%, 90% and 80% for 2 minutes each time.

The slides were washed in PBS (pH 7.6) for 5 minutes and the sections incubated with 3% (v/v) hydrogen peroxidase for 10 minutes at 37°C to block endogenous peroxidase. After washing with PBS, the slides were dried by blotting the area surrounding the wells. Fifty microlitres of diluted MAb was applied to the respective wells and incubated in a humidified chamber at room temperature for 1 hour.

The slides were washed twice in PBS for 5 minutes each time. The Labelled Streptavidin Biotin (LSAB) staining method was employed. The area surrounding the wells were dried and 50 µl of biotinylated antibody (Dako Co.) was applied to each well and incubated in a humidified chamber for 10 minutes at 37°C. The slides were washed in PBS for 5 minutes and dried. Fifty microlitres of peroxidase conjugated streptavidin (Dako Co.) was added and incubated at 37°C for 10 minutes.

AEC (3-amino 9-ethylcarbazole) substrate solution was prepared with the AEC Substrate System (Dako Co.) by adding 13.5 µl substrate H_2O_2 buffer and 13.5 µl AEC chromogen into 1 ml of substrate buffer. This solution was applied to each well and incubated at 37°C. A rose red stain was observed within 5 minutes. The tissue sections were counterstained with Mayer's hematoxylin for 2 minutes. The slides were

then rinsed in distilled water and immersed in ammonia water for 10 seconds. The slides were mounted with pre-heated glycergel after rinsing with water. The slides were stored in the dark and visualized under light microscopy once the glycergel dried.