

CHAPTER TWO : MATERIALS AND METHODS

2.1 Materials

Chemicals used in this study were of Analar Grade or the highest grade available from BDH Ltd., England; Gibco Laboratory, USA; Merck, Republic of Germany; Fluka Chemika, Switzerland and Sigma Chemical Company, USA.

MacCartney bottles (for peptide synthesis) and other glassware used were soaked in Teepol, thoroughly rinsed in water and subsequently washed three times in distilled water and oven dried before being used.

Solvents and reagents used in peptide synthesis are toxic and flammable or corrosive. Skin and eye contact was avoided by wearing gloves and protective clothing at all times.

2.2 Samples

2.2a Sera Samples

98 sera samples were obtained from the labour ward and the post-natal clinic of University Hospital Kuala Lumpur (UHKL), with the help of Dr. Chua Kaw Bing, from the Dept. of Medical Microbiology, Faculty of Medicine, University of Malaya. The sera were then stored at -20°C until further use. 240 sera samples from individuals aged 1 to 15 years old were obtained from Dr. Mangalam of the Virology Division of the Institute of Medical Research (IMR), Kuala Lumpur. These sera were tested for HHV6B IgA and IgG antibodies.

2.2b Cord Blood Samples

Cord blood samples were collected from the labour ward of UHKL with the help of the house officer on duty. Blood was drained from the umbilical cord into heparinised bottles as soon as possible after birth and subjected to lymphocyte separation within 24 hours at room temperature.

2.2c Saliva Samples

Saliva samples were obtained from healthy volunteers. Saliva was collected using endo-dental Watterollen dental rolls (Germany). The rolls were then spun in salivette (Sarstedt, Germany) at 1500 rpm for 10 minutes to obtain the saliva. Figure 2.1 shows an illustration of the salivette used.

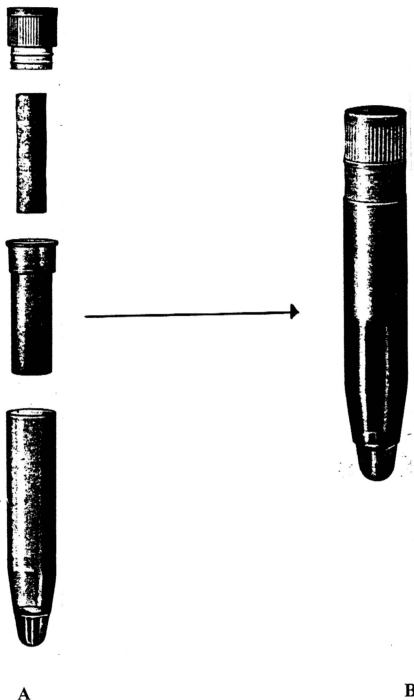


Figure 2.1 : Illustration of salivette (Sarstedt, Germany). The patient was asked to keep a dental cotton roll under his tongue for 10 minutes, then it is removed and put into B. B is then put into A and spun at 1500 rpm for 10 minutes. The saliva would collect at the bottom of valve A.

2.2d Breast Milk Samples

Breast milk samples were obtained from the post-natal clinic of UHKL. The breast milk collected was spun down at 1000 rpm for 10 minutes and the bottom layer was aspirated out into appendorf tubes and stored at 4°C till further use.

2.3 Human Herpes Virus 6 Stock

HHV6 variant B Strain Hashimoto was a gift from Dr. Chua Kaw Bing, of the Dept. of Medical Microbiology, Faculty of Medicine, University of Malaya. The virus stock was stored in polypropylene vials at -70°C. Each vial contained 1ml of HHV6 infected HCBMC, with a minimum of 2000 HHV6 infected cells.

2.4 Materials for Separation, Preparation and Harvesting of Human Cord Blood Mononuclear Cells (HCBMC)

2.4a. Phosphate Buffered Saline (PBS)

80.0 g sodium chloride (Univar, Australia), 2.0 g potassium chloride (Merck), 11.5 g di-sodium hydrogen orthophosphate anhydrous (BDH, England) and 2.0 g potassium potassium dihydrogen orthophosphate (Merck) were dissolved in 1 litre of distilled water. The pH was adjusted to 7.2, and the volume made up to 10 litres with distilled water.

2.4b. Human Interleukin-2 (IL-2)

5 µg of human IL-2 (Gibco, USA) was reconstituted in 50 µl of 100 mM acetic acid (Merck, Germany) supplemented with 0.5% bovine serum albumin (Sigma, USA).

1 μ l of the mixture was then dispensed into each of 50 polypropylene tubes. The tubes were stored in -20°C until further use.

2.4c. Phytohemagglutinin (PHA) Reagent

The lyophilized form of PHA (Gibco, USA) was rehydrated in 10 ml distilled water. Then, 2 ml of the solution was dispensed into each of 5 appendorf tubes (ISC, Utah). The tubes were stored at -20°C till use.

2.4d. RPMI 1640 Cell Culture Medium

One packet (15.19 gm) of RPMI 1640 (Flowlab, Australia) without Hepes (N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid) with L-glutamine (Flowlab, Australia) together with 2 g/L NaHCO₃ (BDH, Analar) and 2 ml penicillin (5000 μ g/ml) and streptomycin (5000 IU/ml) (Flowlab, Australia) were dissolved in 950 ml of sterile distilled water. pH was then adjusted to 7.2 and volume made to 1000 ml. The mixture was filter sterilized through 0.2 micron pore size nitrocellulose membrane and stored at 4°C till use.

2.4e. Supplemented RPMI 1640 Growth Medium

1 μ l of human IL - 2 and 2 ml of PHA were added to 100 ml of 10% heat-inactivated foetal calf serum (FCS) (Flowlab, Australia) supplemented RPMI 1640 culture medium. pH was then adjusted to 7.6 and the mixture filtered using a 0.2 micron pore size nitrocellulose membrane and keep at 4°C.

2.4f. RPMI 1640 Maintenance Medium

100ml of this medium was constituted by 95 ml RPMI 1640 culture medium, 2 ml of FCS and 2 ml penicillin (5000 µg/ml) and streptomycin (5000 IU/ml) (Flowlab, Australia). pH was adjusted to 7.4. The mixture was then filter sterilised and kept at 4 °C.

2.5 Methods for Separation, Preparation and Harvesting of Human Cord Blood Mononuclear Cells (HCBMC)

2.5a Lymphocyte Separation

The following procedure was used to separate lymphocytes from red cells and granulocytes. Cord blood was diluted 1:1 with RPMI 1640 culture medium. 5 ml of the mixture was overlaid onto 3 ml Ficoll-hypaque (density = 1.077) (lymphocyte separation medium) and subjected to centrifugation at 1500 rpm for 30 minutes. The lymphocytes would form a grey coloured layer at the plasma-ficoll interface. The clear upper plasma layer was carefully removed and discarded. Then the lymphocyte band was carefully aspirated and dispensed into a tube containing 5 ml phosphate buffered saline (PBS). The cells were centrifuged at 1500 rpm for 5 minutes. This washing process was repeated twice. After the final wash, the pellet was resuspended in 5 ml pre-warmed growth medium and cultured in 25 ml culture flasks (NUNC) at 37 °C, 5% CO₂ for 2-3 days.

2.5b Infection of HCBMC with HHV6

After 2-3 days, actively dividing and aggregating HCBMC should be seen. The activated HCBMC was spun at 1500 rpm for 10 minutes. The supernatant was discarded

and the pellet washed three times with PBS. After the final wash, the supernatant was removed and 0.5 – 1.0 ml of virus stock was added to the pellet. This was then spun at 1500 rpm, 10 minutes. This was done three times and at each interval, the pellet was resuspended in the supernatant. After the final spin, the supernatant was discarded and the pellet resuspended in 5 ml maintenance medium. The mixture was dispensed into 25ml culture flasks and kept at 37 °C, 5% CO₂ for 3 – 4 days (Chua *et al.*, 1996).

2.5c Cell Harvesting and Preparation of Slides

Harvesting of infected human cord-blood mononuclear cells (HCBMC) was done when 50% of the HCBMC showed cytopathic effect (CPE). This is usually at the 4th day of post-infection when the cells are refractile and significantly enlarged. First, the mixture was centrifuged at 1500 rpm for 10 minutes. The supernatant was then discarded and 5 ml PBS added and centrifuged again. This step was repeated twice. After the final wash, the supernatant was discarded and the pellet resuspended in a small volume (approximately 0.5-1ml) of PBS. The suspension was then dropped onto 10-well Teflon-coated slides, left to dry and then fixed in cold acetone (-10 °C) for 10 minutes, air-dried and were ready for use. The slides could be stored at -20 °C.

2.5d HHV6 negative control slides

The mature lymphocytes, which were left to grow in the CO₂ incubator, are divided into two. One for infection with HHV6 and the other was harvested without infection. The batch harvested without infection was used as HHV6 negative-control slides.

2.6 Indirect Immunofluorescence Assay (IFA) for Detection of IgG and IgA Antibodies Against HHV6 Antigens

Sera were diluted 1 :10 in PBS pH 7.2. Negative and positive controls were always included in the test. Saliva and breast milk samples were not diluted before testing.

20 µl Sera were added to 10-well teflon slides with cells fixed for IFA. The slides were incubated for 45 minutes at 37 °C in a humid chamber. Three washes with PBS followed, each wash taking 3 – 5 minutes. Slides were then allowed to air dry. Wells were not allowed to dry as this could cause non-specific antibody binding. 20 µl of fluorescein conjugated immunoglobulin A (IgA) or IgG (Dako, Germany) were then added onto each well and a second incubation of 45 minutes was carried out. FITC - conjugated to IgG and FITC - conjugated to IgA were both used at 1:20 dilutions.

After incubation, slides were mounted with 1:1 PBS:glycerol solution and then observed under the UV light microscope (Olympus BH2, Japan) using 200x magnification. The presence of HHV6 is indicated by punctate green fluorescence in the nucleus and cytoplasm of infected cells. Any nonspecific fluorescence was cross-checked with HHV6B negative control slides.

2.7 Electron Microscope Observation of HHV6 in Infected HCBMC

Five millilitres of fourth-day HHV6 infected HCBMC culture was spun down at 1500rpm (500 x g) for 10 minutes. The cells were then washed three times in PBS and finally resuspended in 1ml of PBS.

A drop of suspension was placed on a formvar (Sigma, USA) coated copper grid (Agar Scientific Ltd, England). Particles in the suspension were allowed to set for 5 minutes before the excessive fluid was dripped off. The coppergrid, which now had a layer of cells was blotted dry and negatively stained with 1% uranyl acetate (Agar Scientific Ltd, England). Five minutes later, the stain was washed off with distilled water and the coppergrid allowed to air-dry, before being viewed under a CM 12 Transmission Electron Microscope (Philips, Holland) to identify virus particles.

2.8 Peptide Synthesis of the Carboxyl-Terminal of p101 Nucleocapsid Protein of HHV6 Using the Multipin Peptide Synthesis Method

The peptides were derived from the amino acid sequence of a 101K major immunoreactive virion protein of HHV6 variant B strain Z29 from Pellet (1993). The synthesis schedule was generated using the Pepmaker programme. From the p101 protein sequence, which consisted of 858 amino acids, a 334 amino acid sequence (Figure 2.2) was selected and synthesized using the Chiron non-cleavable multipins mimotope mapping technique as 14-residue with 7 amino acid overlap peptide sequences (Geysen *et al.*, 1984; 1987; Houghten *et al.*, 1985, 1986) (refer Appendix 1 and 2)

2.8a Chemistry of Peptide Synthesis

All amino acids recommended for the Multipin Peptide Synthesis kit have their alpha amino acid group protected with the 9-fluorenylmethylcarbononyl (F-moc) group. The amino acid side chains are protected by the following groups :

- a) t-butyl ether (But), for serine, threonine and tyrosine

- b) t-butyl ester (Obut) for aspartic acid and glutamic acid
- c) t-butoxycarbonyl (BoC) for lysine and histidine
- d) 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine and
- e) trityl (Trt) for cysteine.

The protected amino acids are activated *in situ* by addition of the activator , DIC and the additive, 1- hydroxy-benzotriazole (HOBt).

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1:  VSLIKDLRDKDGFRKQKKLDLLGSWTKEKN
31: DKAIVHSREVTGDSGDATETVTARDSPVLR
61: KTKHANDIFAGLNKKYARDVSRGGKGNSRD
91: LYSGGNAEKKETSGKFNVDKEMTQNEQEPL
121: PNLMEAARNAGEEQYVQAGLGQRVNKILAE
151: FTNLISLGEKGIQDILHNQSGTELKLPTEN
181: KLGRESEANVERILEVSDPQNLFKNFKLQ
211: NDLDVSVQSPFRLPNADLSRDLDVSF KDAL
241: DVKLPGNGEREIDLALQKVKAGERETSDFK
271: VGQDETLIPTQLMKVETPEEKDDVIEKMVL
301: RIRQDGETDEETVPGPGVAESLGIAAKDKSVIAS

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Figure 2.2 : Amino acid sequence of the peptides of HHV6 variant B p101 protein consisting of 334 amino acids from the carboxyl terminal (amino acid 524-858). The overlapping peptides synthesized are shown in Appendix 2.

2.8b Selection of Synthesis Regions and Generation of the Synthesis Schedule

The selection of the regions for synthesis was done using MacDNASIS and an Internet Protscale (<http://expasy.hcuge.ch/cgi-bin/protscale.pl>) analysis, based on the method of Hopp and Woods (1981,1983). Both MacDNASIS and Protscale programs require the use of single letter amino acid codes of the protein sequence for analysis. Hydropathy and hydrophilicity plots provide the first basis of antigenicity prediction of a region of interest in a protein, before shorter peptide fragments were synthesized to determine the protein's specific immunodominant epitopes. Besides using the hydropathy plot, the selection was also based on the observation by Neipel *et al.* (1992) who found that the carboxyl terminal of proteins contain more antigenic regions compared to other sites.

The selected sequences of amino acids were entered into the Multipin Peptide Synthesis software and a Gnet schedule was generated. A total of 46 peptides were generated and the schedule also listed out the amounts of amino acids and other chemicals required for each day of synthesis and the wells into which they were to be dispensed (Appendix 2).

2.8c Pre-synthesis Preparation

Before the synthesis was carried out, several procedures had to be carried out. The Chiron multipin peptide synthesis kit containing polyethylene pins attached to pin blocks for amino acid synthesis (Figure 2.3), negative as well as positive control pins with their corresponding monoclonal antibody were all packed in a polyethylene sandwich box. The

boxes were stored at -20°C . Ten 96-well polyethylene reaction trays, together with pin holders were stored at room temperature. Prior to synthesis, the blocks were prepared by labelling them and marking their orientation so that the blocks and trays were aligned. The numbering system of the is shown in Appendix 3.

2.8d Weighing Amino Acids and Activating Chemicals

The next step would be to weigh the amino acids required for each coupling. The weighing was done away from light. Amino acids, HOBt (1-hydroxybenzotriazole) (Sigma Chemical Co., USA) and DIC (diisopropylcarbodiimide) (Sigma Chemical Co., USA) were weighed into MacCartney bottles. Weighed amino acids were stored at 4°C . Cross contamination of amino acids was avoided by rinsing the spatula in alcohol after each weighing process.

2.8e N, N - Dimethylformamide (DMF) Filtration

N,N-Dimethylformamide (DMF) is the preferred solvent for use in F-moc chemistry. The DMF used in the synthesis must be pure and free from amines. This is because in the presence of light at room temperature, DMF slowly decomposes liberating dimethylamine which is most efficient at cleaving the highly labile F-moc protecting group. To avoid this happening at the wrong time during synthesis, it is necessary to use freshly purified DMF. DMF is purified using a freshly packed column containing aluminium oxide (2/3 acidic, pH 4.5 ; 1/3 basic, pH 9.5, whereby the basic alumina is placed at the top of the column).

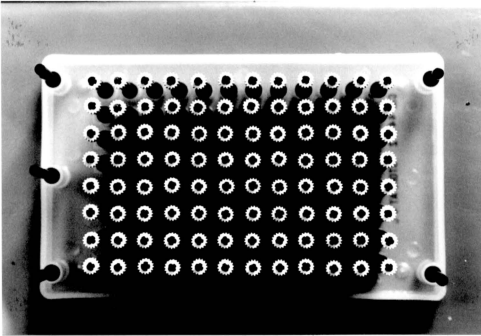


Figure 2.3 : polyethylene pins arranged in a 8 x 12 configuration corresponding to the wells of a 96 well microtitre plate.

2.8f Deprotection of Pins

Deprotection was carried out immediately as follows:

- a) Pins were placed into 20% (v/v) piperidine : DMF solution for 20 minutes at room temperature. The solution should be sufficient to immerse gears fully.
- b) Pins were removed, excess liquid shaken off and then immersed again in DMF bath for 2 minutes at room temperature , with agitation. Once again, solution should cover gears fully.
- c) Pins were removed and excess liquid shaken off. Pins were then soaked in methanol (full immersion) for 2 minutes. This washing process was repeated twice with fresh methanol each time.
- d) Pins were then left to dry for at least 30 minutes at room temperature.

While the pins are drying, amino acid solutions required for the coupling step can be prepared and dispensed as needed.

2.8g Amino Acid Couplings

Amino acids for a coupling reaction were taken out of storage and allowed to thaw to room temperature. The amount of N-hydroxybenzotriazole (HOBt) (Chiron, Australia) and diisopropylcarbodiimide (DIC) needed were measured and dissolved in purified N,N-Dimethylformamide (DMF) (Sigma, St. Louis, USA) (refer Appendix 2) according to the amount in the synthesis schedule. Abbreviations of amino acids can be referred to in Appendix 1. A specific amount of DMF was added into DIC and the solution was vortexed to ensure homogeneity. Homogenous HOBt solution was then added to the amino acids and vortexed until the amino acids dissolved fully. DIC, the

amino acid activator was added last. The amino acids were dispensed following a specific sequence as shown in Appendix 1.

Once dispensed, the pins are placed on trays in the correct orientation and left for coupling. Couplings were done at a 4 - hour to overnight incubation period.

2.8h Washing of Pins

Once the coupling reaction was done, the pins were removed from the reaction tray and washing carried out according to the steps below:

- a) Pins were immersed to half their height in methanol bath, and agitated for 5 minutes. Excess methanol was flicked off and pins allowed to air-dry for 2 minutes.
- b) Immersion in DMF covering half the height of the pins, for 5 minutes, with agitation.
- c) The next cycle, beginning with the deprotection, can commence immediately after this washing step.

The deprotection, washing, coupling and washing step were repeated sequentially until all the amino acids were coupled.

2.8i Acetylation of Terminal Amino Groups

The N-terminus of each peptide was acetylated to remove the charge associated with a free terminal amino group. Thus, after the final coupling, the pins were washed and deprotected, and then subjected to the acetylation of amino terminal groups. The pins were placed into a reaction tray containing 150 μ l/well of the following mixture:

DMF : acetic anhydride : N - ethyldiisopropylamine
193 : 6 : 1 (v/v/v)

The tray was placed in a sealable polypropylene bag or container. This reaction was allowed to proceed for 90 minutes at room temperature, after which the pins were removed and washed in methanol bath for 15 minutes. The pins were then allowed to dry for another 15 minutes.

2.8j Side-chain Deprotection

After the pins have dried, they were subjected to side-deprotection. All protecting groups used to protect side-chain functionalities during synthesis had to be removed from the synthesized peptides before they were used. This was accomplished by treating the pins with the following cleavage mixture in polypropylene baths 150 minutes at room temperature.

Trifluoroacetic acid : anisole (v/v)
19 : 1

Upon removal from the deprotection bath, the pins were immediately fully immersed in methanol for 10 minutes. The blocks were shaken to remove excess methanol and then soaked again in 0.5% acetic acid (0.5 ml acetic acid per 100ml) in methanol/purified water (1 : 1 v/v) for 60 minutes. This was followed by two washes in fresh methanol, two minutes each. Excess solvent was flicked off and the blocks left to dry overnight. The pins were then ready to be used for ELISA testing . A schematic presentation of the steps involved in peptide synthesis is shown in Figure 2.4.

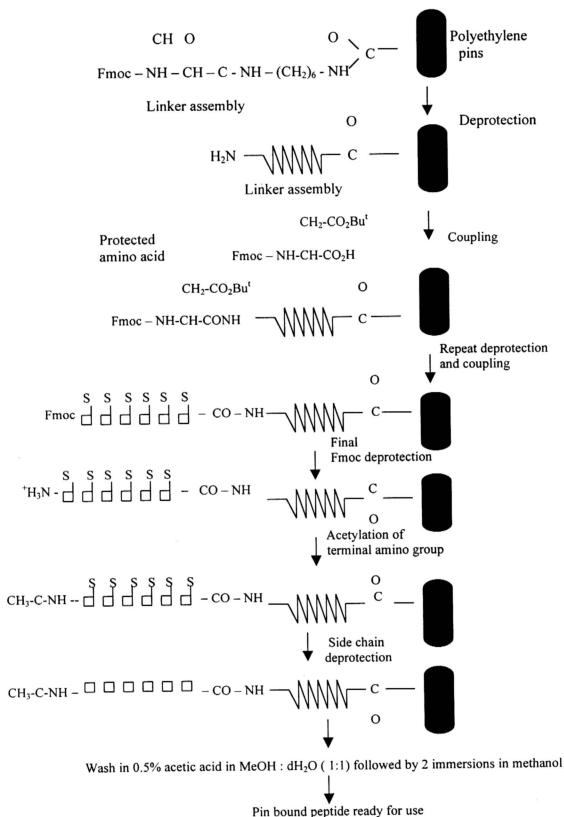


Figure 2.4 : Schematic presentation of the steps involved in peptide synthesis. Fmoc is the N-terminal protecting group. Bu^t and S represent side chain protecting groups and □ represents the individual amino acids.

2.9 Enzyme-Linked Immunosorbent Assay (ELISA) on Samples Against the p101 Synthesized Peptides

The following adaptation of the ELISA protocol was used with the multipin system to scan the synthesized peptides and define specific epitopes.

A critical control in such an experiment is to show that the conjugate itself does not bind directly to the peptide. The first test of any set of pin bound peptides should be with the conjugate alone, to eliminate direct binding by conjugate as a source of artefact.

Pre-coating of pins ('blocking' step)

Pre-coating of pins reduces non-specific binding thus giving a better signal-to-background ratio.

The pre-coat buffer was then prepared by adding

2% w/v bovine serum albumin (fraction V, United States Biochemicals, USA)

0.1% v/v Tween 20 (Sigma Chemical Co., USA)

0.1% w/v sodium azide in 0.01M phosphate buffered saline pH 7.2 (PBS).

To pre-coat the pins, 150 μ l of the pre-coat buffer was dispensed into each well of microtitre plates. The pins, with gears fully immersed in the pre-coat buffer were incubated for 60 minutes, at room temperature on a shaker platform (Belly Dancer, USA). After this hour, incubation with primary antibody was carried out.

Incubation with primary antibody

One hundred and fifty microlitres of human serum diluted 1:100 in pre-coat buffer was dispensed into appropriate wells of the microtiter plates. Pins were incubated

overnight at 4°C. The following day, the pins were washed 4 times in 0.01M PBS (pH 7.2). Fresh PBS was used each time and each wash was for 10 minutes at room temperature with agitation.

Saliva were tested at 1:10 dilution while breast milk were tested at a dilution of 1 : 50. HRP anti-IgA used for both saliva and breast milk samples was diluted at 1:500.

Conjugate Reaction (secondary antibody)

The conjugate diluent used consisted of:

1% v/v sheep serum

0.1% v/v Tween 20 (Sigma Chemical Co., USA)

0.1% w/v sodium caseinate (Sigma Chemical Co., USA) in 0.01M PBS pH 7.2

Peroxidase-labelled affinity purified anti-human antibody (anti-human IgG or anti-human IgA) was diluted to a dilution of 1:1000 with the above conjugate diluent and was dispensed at 150µl /well. The pins were then allowed to incubate for 1 hour on a shaker , at room temperature. After incubation , the pins were washed 4 times in 0.01M PBS (pH 7.2). Fresh PBS was used each time and each wash was for 10 minutes at room temperature with agitation.

Substrate reaction

Substrate buffer solution

The substrate buffer solution for chromogenic development was prepared using 7.1g Na₂HPO₄ anhydrous (Fluka, Switzerland) and 8.4g citric acid monohydrate (Ajax Chemicals, Australia) which were dissolved in 500ml of distilled water at room

temperature. The pH was adjusted to 4.0 with 1.0M Na₂HPO₄ or 0.8M citric acid when necessary. The buffer was stored at 4°C and the required aliquots brought to room temperature before use each day. The substrate buffer was used within two weeks.

Orthophenylenediamine (OPD) substrate solution

OPD (Orthophenylenediamine) (Sigma Chemical Co., USA) was dissolved in substrate buffer solution at room temperature to a concentration of 0.5mg/ml. Hydrogen peroxide (BDH, UK) solution was added to give a final concentration of 0.01% w/v after all the OPD had completely dissolved in substrate buffer solution.

Substrate solution was prepared immediately before use. 150µl /well of the above substrate solution was dispensed into ELISA microtitre reading plates (i.e., flat-bottom polystyrene plates) (NUNC Maxi Sorp, Danmark) and left for incubation on a shaker table (Belly Dancer, USA) for 35 minutes at room temperature.

The reaction was stopped by removing the pins from the ELISA microtitre plates containing the substrate solution. Figure 2.5 shows the chromogenic development as seen in the wells of the microtiter plate. The plates were read on a Dynex MRX ELISA reader as soon as possible after incubation. The optical density was read at 490nm and 630nm in a dual wavelength mode.

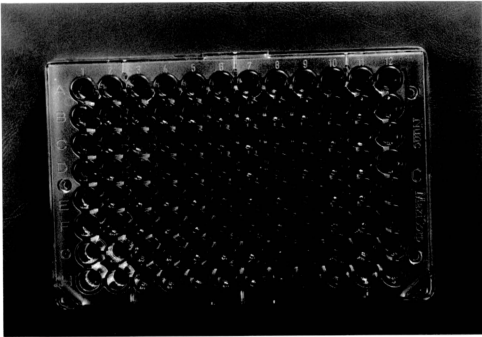


Figure 2.5 : ELISA plate showing the chromogenic development in wells following pin-ELISA.

Antibody removal (Disruption procedure)

A major benefit of the multipin system is that peptides can be reused. The peptides are covalently coupled to the pins which can be treated such that all bound antibodies are removed. This is done by sonicating the pins for 10 minutes at 65°C.

The disruption buffer used for antibody removal contained 1% w/v sodium dodecyl sulphate (SDS) (Fluka, Switzerland) in 0.1M phosphate buffer (PBS). pH was adjusted to 7.2. 2-mercaptoethanol was added to a final concentration of 0.1% v/v.

The buffer was preheated to 60°C. Pins were then placed downward into the disruption buffer in the sonication bath after the temperature had equilibrated at 60°-65°C and was sonicated for 10 minutes. The blocks were then rinsed twice in hot distilled water (60°C) for 30 seconds. The blocks were later placed in a swirling water bath at 60°C for 30 minutes. Excess water was shaken off the pins and an immersion in hot methanol (60°C) for 15 seconds was carried out. The blocks were allowed to air dry and were ready for reuse. When not used immediately the blocks were stored in a sealed container with desiccant at 4°C.

Before discarding the used disruption buffer, hydrogen peroxide was added at a concentration of 2 ml/liter and allowed to stand for 5 minutes. This is done to destroy any remaining 2-mercaptoethanol. The effectiveness of antibody removal is checked by repeating the test for conjugate activity.

Controls

The multipin peptide synthesis kit contains its own control pins with a pre-synthesized sequence and control antibody for these pins. These pins serve as a check on the ELISA procedure.

Peptides with the sequence of PLAQ will give a positive ELISA result with the and peptides with the sequence of GLAQ will give a negative ELISA result with the control antibody.