# CHAPTER 2

# MATERIALS AND METHODS

"Everything has its beauty but not everyone sees it"

**Confucius**

# Materials and Methods

## 2.1. Materials

Note: All chemicals were of Analar grade or of the highest grade available from BDH Ltd., England; Fluka Chemika, Switzerland; Gibco Laboratory, U.S.A.; Merck, Germany; Sigma Chemical Co., U.S.A. and United States Biochemical (USB) Corp., U.S.A.

a) The common chemicals included acetone, ethanol, formamide, glacial acetic acid, glucose, glycerol, hydrochloric acid, isoamyl alcohol, methylated spirit, phenol, pottasium chloride, sodium acetate, sodium chloride, disodium hydrogen phospate (anhydrous), sodium dihydrogen phosphate (monohydrate), sodium dodecyl sulphate (SDS), sodium hydroxide and Trizma base (Tris[hydroxymethyl] aminomethane), methanol (MeOH).

b) Chemicals used for the peptide synthesis procedure included N, N-dimethylformamide, piperidine, acetic anhydrate, triflouroacetic acid (TFA), ethanedithol (EDT), 1-hydroxy-benztriol (HOBt), triethylamine (TEA), anisole and diisopropyl-carbodiimide (DIC).

c) Additional chemicals used for the (modified) ELISA procedure included Bovine Serum Albumin (BSA), Tween- 20, sodium azide, sheep serum, sodium caseinate, citric acid monohydrate diammonium 2,2'-azino-bis[3-ethylbenz-thiazoline-6-sulfonate] (ABTS).

### 2.1.2 Reagent solutions for ELISA

a) PBS 10X

0.1M phosphate buffered saline (PBS) is used to prepare working strength PBS for use in ELISA tests.

53.7 g $Na_2HPO_4.2H_2O$ (or 42.8 $Na_2HPO4$ anhydrous or 107.44g $Na_2HPO_4.12H2O$)

15.6 g $NaH_2PO_4.2H_2O$

340 g NaCl

Dissolve the salts in hot distilled water to give a final volume of 4 liters. Mix thoroughly and allow the solution to cool to room temperature. Adjust the pH to 7.2 with either 50% w/v NaOH or concentrated (37%w/v) HCl. This solution is ten times the working strength and can be stored at room temperature for short periods, or in a refrigerator over extended periods. When required for use, the PBS 10X stock solution is diluted 1/10 with distilled water.

b) pre-coat buffer

This buffer is used to pre-coat pins, so as to reduce non-specific binding, to give a better signal to background ratio in ELISA assays, and is also used as the diluent for the test antibodies.

2% w/v Bovine Serum Albumin (BSA)

0.1 % v/v Tween 20

0.1 % w/v sodium azide

0.01 M phosphate buffered saline(PBS) pH 7.2

This solution can be store in the refrigerator for 24 hours, or freezed at -20$^0$C for long term storage.

c) conjugate diluent

This is the diluent for the goat anti-species conjugate in the ELISA.

1% w/v sheep serum

0.1% v/v Tween 20

0.1% w/v sodium caseinate (USB)

0.01 M phosphate buffered saline(PBS) pH 7.2

This solution can be stored in a refrigerator for up to 24 hours, and freezed at -20$^0$C for long term storage.

d) substrate buffer solution

This buffer is used as the solvent for the chromogenic substrate in ELISA.

35.6 g $Na_2HPO_4.2H_2O$ (or 28.4 g $Na_2HPO4$ anhydrous)

33.6 g citric acid monohydrate

The salts are dissolved in hot distilled water and made up to 2 litres with distilled water at room temperature. The pH is adjusted to 4.0 if necessary with 1M $Na_2HPO_4$ solution or 0.8 M citric acid. The substrate buffer should be stored in the refrigerator and the amount required should be brought to room temperature before use each day. It should be used within 2 weeks of preparation and checked for signs of contamination before use each time.

e) ABTS Substrate Solution

This is the chromogenic substrate solution for the horseradish peroxidase conjugate to detect antibodies, and should be prepared immediately before use. Equilibrate the substrate buffer solution to room temperature.

Dissolve 0.5 mg of diammonium 2, 2'-azino-bis[3-ethylbenz-thiazoline-6-sulfonate] (ABTS) in substrate buffer. When the ABTS has completely dissolved, add sufficient hydrogen

peroxide solution (35%w/w-"120vol") to give a final concentration of 0.01% w/v.

B) Micropipette tips and McCartney bottles were autoclaved before use.

C) Multipin Peptide kits were obtained from Chiron Technologies, Pty, Ltd. (Clayton, Victoria, Australia.)

### 2.1.3 Serum Samples and Monoclonal Antibodies

A) All serum samples designated "typhoid positive" were obtained from patients diagnosed with typhoid fever by a positive culture of *Salmonella typhi* (Appendix I) at the University Hospital, Kuala Lumpur.

B) Serum samples designated "normal sera" were obtained from healthy blood donors at the Blood Bank, University Hospital, Kuala Lumpur.

C) The monoclonal antibody against *Eschericia coli* GroEL (SPA 870) was purchased from StressGen Biotechnologies Corp., Vancouver, BC, Canada.

2.2 Methods

### 2.2.1 Peptide Synthesis Procedure

1. Selection of Synthesis Regions and Generation of the Synthesis Schedule
2. Pre-synthesis Preparation
3. Weighing Amino Acids and Activating Chemicals
4. Filtering DMF
5. F-Moc Deprotection and Washing of Synthesis Pins
6. Coupling the N-α-Fmoc – Protected Amino acids
7. Subsequent Coupling and Washing
8. Acetylation of Terminal Amino Groups
9. Side –Chain Deprotection

### 2.2.2 ELISA Testing

a) Conjugate Test
b) Primary Antibody Test

### 2.2.3 Removal of the bound antibody ("Disruption")

### 2.2.1 Peptide Synthesis Procedure

#### 2.2.1.1 Selection of Synthesis Regions and Generation of the Synthesis Schedule

**a) Selection regions fo GroEL to be synthesized.**

9-mer overlapping nonapeptides were synthesized based on the highly hydrophilic regions of GroEl, the 60kDa heat shock protein of *Salmonella typhi*. The amino acid sequence was based on the published amino acid sequence of GroEL (Lindler and Hayes , 1994) (Figure 2.1).

With this data, the highly hydrophilic regions of GroEL were determined from Hopp and Wood's hydrophobicity plot (Stover et al., 1990) or using the ExPasy program accessed via the University of Geneva www server.

The peaks below the x-axis in this plot show the hydrophilic regions. The hydrophilic regions were then picked out (Figure 2.3) and considered as one continuous region to be synthesized.

```
1.    ATGGCAGCTAAAGACGTAAAATTCGGTAACGACGCTCGTGTGAAAATGCTGCGCGGCGTA
      M  A  A  K  D  V  K  F  G  N  D  A  R  V  K  M  L  R  G  V  20
61.   AACGTACTGGCAGATGCAGTGAAAGTAACCCTCGGTCCGAAAGGCCGTAACGTGGTTCTG
      N  V  L  A  D  A  V  K  V  T  L  G  P  K  G  R  N  V  V  L  40
121.  GATAAATCTTTCGGTGCGCCGACTATCACTAAAGATGGTGTTTCCGTAGCGCGTGAAATC
      D  K  S  F  G  A  P  T  I  T  K  D  G  V  S  V  A  R  E  I  60
181.  GAGCTGGAAGACAAGTTTGAAAACATGGGCGCGCAGATGGTGAAAGAAGTTGCCTCTAAA
      E  L  E  D  K  F  E  N  M  G  A  Q  M  V  K  E  V  A  S  K  80
241.  GCGAACGACGCTGCAGGCGACGGCACCACCACCGCGACCGTACTGGCGCAGTCCATCATT
      A  N  D  A  A  G  D  G  T  T  T  A  T  V  L  A  Q  S  I  I  100
301.  ACCGAAGGCCTGAAAGCCGTTGCTGCGGGCATGAACCCGATGGACCTGAAACGTGGTATC
      T  E  G  L  K  A  V  A  A  G  M  N  P  M  D  L  K  R  G  I  120
361.  GACAAAGCGGTTGCTGCTGCGGTTGAAGAGCTGAAGGCGCTGTCCGTACCGTGCTCCGAC
      D  K  A  V  A  A  A  V  E  E  L  K  A  L  S  V  P  C  S  D  140
421.  TCTAAAGCGATTGCTCAGGTAGGTACTATTTCCGCTAACTCCGACGAAACCGTAGGTAAA
      S  K  A  I  A  Q  V  G  T  I  S  A  N  S  D  E  T  V  G  K  160
481.  CTGATCGCGGAAGCGATGGATAAAGTCGGTAAAGAAGGCGTCATCACTGTTGAAGACGGT
      L  I  A  E  A  M  D  K  V  G  K  E  G  V  I  T  V  E  D  G  180
541.  ACCGGTCTGCAGGACGAACTGGACGTGGTTGAAGGTATCGAGTTTGACCGTGGCTACGTG
      T  G  L  Q  D  E  L  D  V  V  E  G  M  Q  F  D  R  G  Y  L  200
601.  TCTCCTTACTTCATCAACAAGCCGGAAACTGGCGCAGTAGAACTGGAAAGCCCGTTCATC
      S  P  Y  F  I  N  K  P  E  T  G  A  V  E  L  E  S  P  F  I  220
661.  CTGCTGGCTGATAAGAAAATCTCCAACATCCGCGAAATGCTGCCGGTTCTGGAAGCCGTT
      L  L  A  D  K  K  I  S  N  I  R  E  M  L  P  V  L  E  A  V  240
721.  GCAAAAGCAGGCAAACCGCTGCTGATCATCGCTGAAGATGTTGAAGGCGAAGCGCTGGCT
      A  K  A  G  K  P  L  L  I  I  A  E  D  V  E  G  E  A  L  A  260
781.  ACCCTGGTAGTGAACACCATGCGTGGCATCGTGAAAGTGGCTGCGGTTAAAGCACCGGGC
      T  L  V  V  N  T  M  R  G  I  V  K  V  A  A  V  K  A  P  G  280
```

**Figure 2.1**: **The nucleotide sequence of *Salmonella typhi groEL*** (reproduced from Lindler and Hayes, 1994). The numbers on the left indicate nucleotide position and the numbers on the right indicate amino acid positions in the encoded amino acid sequence. This sequence has been deposited in the GenBank database and given accession number U01039.

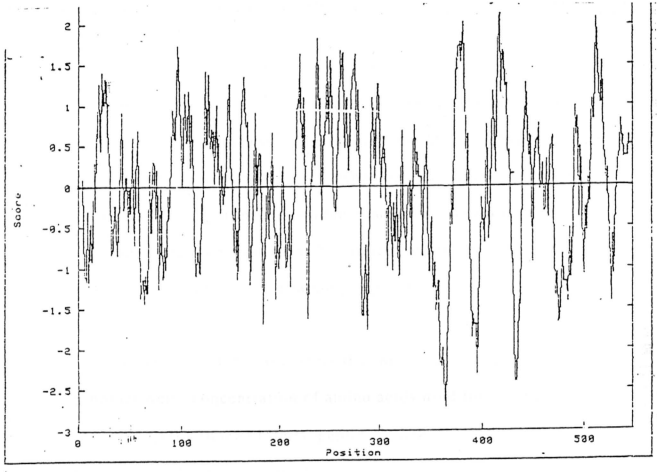


**Figure 2.2 Hydophobicity Plot ( Hopp and Wood) generated based on the *groEL* sequence ( Lindler and Hayes, 1994 ) using the ExPasy program (University of Geneva www server ).**

KDKFGNDRKRGNDKTGKGRNDKSGTTKDGSREEEDKENGQKESKNDGDGTTTTQSTEGK
GNDKRGDKEEKSCSDSKQGTSNSDETGKEDKGKEGTEDGTGQDEDEGQDRGYSYNKETG
EESDKKSNREEKGKEDEGETNTRGKKGGDRRKQDTTGGTSEEGEEKTEDGQKRNKDTTT
DGGEEQGRQRQQEETSDYDREKQERKGGKGTEEKEKKREDHTREEGGGGRSKDKGQNED
QNGKRERQNCGEESNT

**Figure 2.3 : The continuous hydrophilic region used for synthesis**

## b) Generating The Synthesis Schedule

The amino acid sequence deduced after eliminating the hydrophobic regions was then entered into the PepMaker application in the software supplied with the Multipin Peptide Syntesis kit supplied by Chiron Technologies (Clayton, Victoria, Australia). The appropriate residue numbers of the amino acid sequence were then appropriately entered into the program. The program generated 122 overlapping peptides to be synthesized in duplicate.

Other important parameters that were selected in the program were: concentration of amino acids used for synthesis (60mM), well volume (150µl), peptide length (9-mer), overlap (one) and number of copies (duplicate).

Once all the parameters were entered and checked, the program generates a synthesis schedule for 9 days, where one amino acid is synthesized per day and 9 days were required for synthesizing 9-mer length peptides.

The synthesis schedule lists the amount of individual amino acids and other chemicals required for a day's synthesis and also the well positions ( on the reaction tray) for adding the activated amino acid ester solutions ( Appendix 3 ).

### 2.2.1.2 Pre-synthesis Preparation

a) Storage: The peptide synthesis kit from Chiron Technologies consisted of polyethylene pins, blocks, reaction trays, sealable containers, control pins, control antibodies (all of which were stored at 4°C) and the peptide synthesis software (stored at room temperature, away from direct sunlight or direct heat sources).

b) The success of the synthesis depended heavily on clean, non-contaminated and properly stored polyethylene pins . Hence, the pins were properly stored in sealed containers at all times and surgical gloves were worn at all times when handling the pins.

c) Orientation of pins on blocks : The pins were fitted tightly onto "blocks" to make up a 8 X 12 configuration (Figure 2.4) corresponding to that of a standard microtiter plate. To ensure easy identification of blocks and proper orientation of plates and pins, adhesive labels were used.

d) Pin and Reaction Tray Numbering System

The numbering system of the wells used by the synthesis program did NOT conform to the numbering and lettering of a standard ELISA plate ( Figure 2.4). The system used is illustrated in Figure 3 below. Thus, reference number A1 (1,2) on the

synthesis schedule would refer to block A, row 1, columns 1 and 2. A1(1,2) would be wells H1 and G1 using the standard ELISA plate nomenclature. The order in which the peptides are synthesized goes from 1(1) to 12(1), then back to 1(2) to 12(2) and so on.

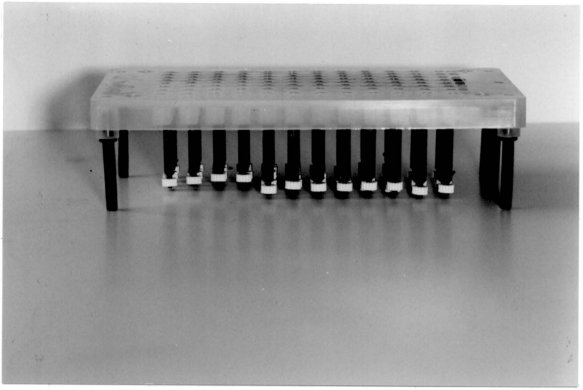

**Figure 2.4 The pins, fitted onto a block, make an 8 X 12 configuration.**

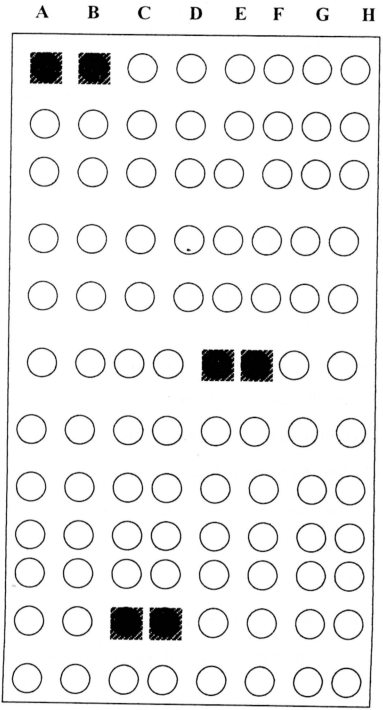


**Figure 2.5 Numbering system used by the Multipin Peptide Synthesis software.** Wells are identified by a letter which represents the block, followed by the row number, which is in turn followed by the column number(s) in the parentheses. For example in block A shown here, the wells shown in the figure as filled are identified as A1(1,2) , A6(5,6) and A11(3,4).

Note: All solvents and reagents used in peptide synthesis are toxic and many are flammable and / or corrosive. In all instances, skin and eye contact were avoided, and all work involving these reagents was done in a fume hood.

### 2.2.1.3 Weighing Amino Acids and Activating Chemicals

Based on the synthesis schedule, F-moc (fluorenylmethyloxycarbonyl)- protected amino acids (Cambridge Research Biochemicals, UK), HOBt (1-hydroxybenzotriol) (Sigma Chemical Company, USA) and DCC (dicyclohexylcarbodiimide) (Sigma Chemical Company, USA) were weighed for the whole synthesis into universal glass bottles. The bottles were thoroughly cleaned and the bottle caps and inserts are inert to any of the reagents and solvents used. During the weighing process, care was taken to avoid cross-contamination of amino acids by rinsing the spatula in ethanol between weighings and by making sure that caps were replaced onto the correct bottles after weighing.

### 2.2.1.4 F-Moc Deprotection and Washing of Synthesis Pins.

The pins are supplied ready for use, and fit onto a "block", configured to fit onto a 96-well microtitre plate (Figure 2.6). However, they must be F-moc de-protected prior to the commencement of synthesis. The N-F-moc protecting group is extremely base-labile and is readily removed by treatment with piperidine in DMF. This was done by placing the blocks of pins in a bath (polyethylene "sandwich" box) containing 20% (v/v) piperidine (Sigma Chemical Co., USA) in DMF for 30 minutes at room temperature. Care was taken to ensure that there was enough solution in the bath to cover the pins up to at least half their height.

Following this, the blocks were then removed form the solution and the excess liquid shaken off, before being placed to wash in a DMF bath. Excess DMF was shaken off and the blocks were subsequently immersed completely in a methanol bath for 2 minutes. The blocks were then washed again, this time with agitation, in a second methanol bath, the methanol coming up to at least half the height of the pins. This washing step was repeated twice before the blocks were finally removed and allowed to air dry.

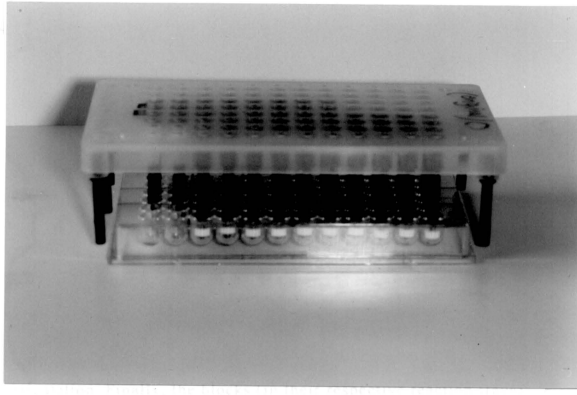

**Figure 2.6 The pins fit onto a "block", configured to fit onto a 96-well microtitre plate**

### 2.2.1.5 Coupling the N-α-Fmoc – Protected Amino acids

HOBt and DCC solutions were prepared by adding volumes of fresh DMF, as indicated in the daily synthesis schedule (Appendix 4). Specified volumes of HOBt/DMF solution were added to the individual amino acids as indicated. The amino acid preparations were completely dissolved before adding the DCC/DMF solution to activate the individual amino acid solutions. Having done this, the activated amino acid solutions were then dispensed to their respective wells of the reaction trays. The air-dried blocks were washed in fresh DMF for 5 minutes before positioning the pins in the reaction trays containing the activated amino acid solutions in the correct orientation. Finally, the blocks (in their respective reaction trays) were placed in a sealable container, and left overnight in the fume hood at room temperature.

### .2.1.6 Subsequent Coupling and Washing

After the overnight incubation to couple the first amino acid, the blocks of pins were taken off the reaction tray to stop the coupling reaction and processed. Placing them first in a DMF bath (to at least half the height of the pins), the blocks were washed for 2 minutes with agitation. Excess DMF was then

shaken off and the blocks were subsequently placed in a fresh methanol bath and washed with agitation for 10 minutes. This methanol wash was repeated twice with fresh methanol being used each time. The blocks were then allowed to air dry for 30 minutes. After this, the pins were then de-protected as described above and the next amino acid coupled as indicated by the synthesis schedule using the method descibed above. The F-moc deprotection, washing, coupling and washing steps were repeated until all the amino acids were coupled (to the required length of peptides were synthesized on the pins). Nine days were needed to synthesize the nonapeptides.

### 2.2.1.7 Acetylation of Terminal Amino Groups

Upon completion of the 9$^{th}$ day of synthesis, the N-terminus of each peptide was routinely acetylated in order to remove the charge associated with a free terminal amino group. The blocks of pins were removed from the reaction trays (after the last amino acid was coupled) and washed as mentioned in section 2.2.1.6 above. The pins were F-moc deprotected and washed, and then placed into trays containing 150µl/ well of the following acetylation mixture:

DMF: acetic anhydride : triethylamine 50:50:1 (v/v)

The trays containing the pins were placed in a sealable container and the reaction allowed to proceed for 90 minutes at $25^0$C. After this period, the blocks of pins were removed and washed in a methanol bath for 15 minutes, followed by air drying for at least 15 minutes. The peptides bound to the pins now are ready for side-chain deprotection.

### 2.2.1.8 Side –Chain Deprotection

All protecting groups used to protect side-chain functionalties during synthesis must be removed from the synthesized peptides prior to ELISA testing. This was accomplished by treating the pins with the following cleavage mixtures:

Trifluoroacetic acid: ethanediol: anisole

95 : 2.5 : 2.5 (v/v)

The pins were treated in a polypropylene bath for 4 hours at $20^0$ C. After removing the blocks from the deprotection bath, the pins were then allowed to air dry for 10 minutes, and then sonicated in 0.1% HCl methanol/ distilled water (1:1) for 15 minutes (Figure 2.7). Following this, the pins were allowed to air dry. The blocks were stored in a sealable container with a dessicant at $4^0$C till needed. The general scheme for synthesizing peptides is summarized in Figure 2.7 below.

### 2.2.2 ELISA Testing

The ELISA protocol adapted here has been tested successfully with the Multipin peptide system and was designed for horseradish peroxidase- labelled anti-species antibodies with ABTS as the chromogenic substrate. (The pins on each block fit directly into a standard 8 X 12 ELISA plate as shown in Figure 2.8)

#### 2.2.2.1 Conjugate Test

The conjugate used in the ELISA protocol is an antibody, and can bind directly to the peptides. Thus the reactivity of the peptides with the conjugate has to be checked before testing with the primary antibody is carried out. This step is more important after one cycle of screening with a primary antibody (here, typhoid positive sera, the "normal sera" and the monoclonal antibody) and the "disruption" procedure carried out. The conjugate test performed then will indicate whether "disruption is complete, i.e. whether all the primary antibody bound to the peptides on the pins has been removed.

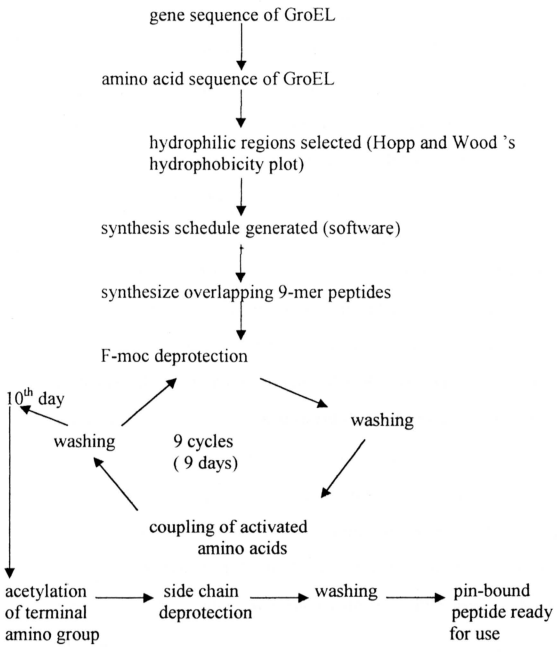


**Figure 2.7 General scheme for synthesizing overlapping 9-mer peptides of GroEL based on the multipin peptide synthesis technique**

Prior to testing the conjugate dilution has to be optimized. Different concentrations of conjugate are tested with the assay. The optimum concentration is found when a plateau is reached upon increasing the concentration of the conjugate. This is the lowest concentration of the conjugate which gives the highest sensitivity of the assay.

The conjugate test is started off with pre-coating the pins (to reduce non-specific binding). 200µl of pre-coat buffer is dispensed into the wells of a microtitre plate. The pins are placed in the wells and incubated for 60 minutes at room temperature on a shaker table. After 60 minutes, the pins are removed from the pre-coating buffer and flicked to rid them of any excess buffer.

The appropriate conjugate dilution is prepared and 200µl of the solution is dispensed into microtitre plated before the pins are incubated for 60 minutes, with agitation. When the time is up, the pins are removed form the conjugate solution and washed four times (10 minutes each time) with fresh PBS used each time in a bath.

The substrate (ABTS) is prepared fresh (for each assay). 200µl of the substrate is dispensed into the wells of an ELISA reading plate. The plates are checked to ensure correct labeling and the blocks are oriented correctly in the plates. Incubation proceeds at room temperature on a shaker table. the reaction is

stopped by removing the pins from the solution and the ELISA plates are then read at 405 nm in an ELISA reader. If the ELISA reader is capable of a "dual wavelength" mode of operation, the optical density at 492 can be subtracted to correct for background. Background here refers to color present in the substrate solution before use and the optical defects of individual wells of the ELISA plate.

#### 2.2.2.2 Primary antibody test

The pins are first "blocked" with pre-coating buffer by loading 200µl of pre-coating buffer. The pins are then removed from the pre-coating buffer and given a good flick (to remove excess pre-coating buffer).

The primary antibody was first diluted in pre-coating buffer. Though the recommended dilution for hyperimmunized animal sera (analogous to typhoid positive sera) was 1: 5000, optimization of the assay showed 1:10000 to be a better dilution and this was used throughout for screening the typhoid positive sera and the "normal sera". The monoclonal antibody though, was diluted at 1: 5000.

200µl of the primary antibody is dispensed into the wells of a microtitre plate before the pins are carefully placed in the

wells and allowed to incubate overnight at room temperature on a shaker table.

The next day the pins are removed from the plates and washed four times in a bath of 0.01M PBS (pH 7.2) , using fresh PBS for each wash.

Subsequently, the pins are subjected to the conjugate test as in 2.2.2.1 above. The pins are washed after the conjugate test, then subjected to the substrate reaction. The reaction is stopped after 10 minutes if the color development is fast but all attempts were made during optimization of the conjugate test to ensure standard reading of the substate reaction after 45 minutes incubation.

### 2.2.2.3 Quality control

To measure the quality of the synthesis, the reactivity of the positive control (PLAQ) and the negative control (GLAQ) is measured. Each individual block has control peptides synthesized (as indicated in the schedule), hence this will provide a means of assesing the quality of the synthesis of each block. In addition to that two sets of control peptide pins are supplied with the kit, one set having the positive control sequence PLAQGGGG and the other the negative control sequence GLAQGGGG, differentiated

from each other by their red and green colors, respectively. The monoclonal antibody provided with the kit (towards the controls), reacts with the PLAQ but not the GLAQ.

### 2.2.3 Removal of the bound antibody ("Disruption")

This is the major advantage of the Multipin System – the peptides can be reused after testing. The covalent coupling of the peptides to the pins allows for harsh and rough handling of the pins during the assays.

#### 2.2.3.1 Materials required

0.1M phosphate buffer with 1% w/v sodium dodecyl sulphate (SDS), pH 7.2 at $60^0$C (adjusted with 50% w/v NaOH or orthophosphoric acid)

2-mercaptoethanol

hydrogen peroxide 35% w/w ( "120 vol")

Methanol

Sonication bath

### 2.2.3.2 The "disruption" procedure

The sonication bath is first rinsed thoroughly with distilled water. The 0.1 M phosphate buffer containing 1% SDS is preheated to $60^0$C. 2-mercaptoethanol is added to a final concentration of 0.1% v/v, and the temperature allowed to eqilibriate to between $55^0$C and $65^0$C (higher temperatures would damage the peptides and lower temperatures would lead to incomplete removal of the bound antibody).

The blocks are placed in the bath with the pins downward (Figure 2.7) and sonicated for 10 minutes. The blocks are then removed, and rinsed in distilled water, pre-heated to $60^0$C for 30 seconds. This process is repeated if there are too many bubbles (from the SDS).

Any excess water is shaken off vigorously from the blocks. The blocks are then immersed in hot distilled water at $60^0$C for 30 minutes before being totally immersed in hot methanol (about $60^0$C) for at least 15 seconds. They are then allowed to air dry for over 15 minutes. They are now ready for further testing.

Ideally a conjugate test is done immediately with each block (before further testing) and if the reactivity is found to be

still high after one cycle of disruption, the disruption process is repeated.


**Figure 2.8 The blocks are placed downward with the pins totally immersed in the Disruption buffer in the sonicator**

### 2.2.3.3 Analysis of Results

Absorbance values obtained from the ELISA procedure were manually fed into the "plot" function of the software supplied with the Multipin Peptide Synthesis kit (Chiron Corporation, Victoria). This will allow the generation of plots for each individual serum tested with the pins.

The software also contains a Consensus plot algorithm, which compares the reacitivities of the peptides with the immune sera, the monoclonal antibody, and the normal (non-immune) sera, to identify which peptides give ELISA signals above background values. The algorithm calculates a mean value for the lower half of all the values and adds three times the standard deviation to the mean . This becomes the cutt-off value. All the values above this cut-off value are treated as significant. A Consensus Plot is the obtained, which indicates the peptides where there is significant binding.

### 2.2.4 ELISA with GroEL heat shock proteins

To demonstrate the importance of the GroEL heat shock protein in *Salmonella typhi* infection, an ELISA was done using purified GroEL of E.coli ( StressGen, British Columbia). Basically, the microtitre plate wells were coated with 1μg GroEL/ well (diluted in coating buffer to make up a volume of 100μl/ well) and incubated overnight at 4°C. The plate was then washed 3 times with PBS-Tween 0.05% and 100μl blocking solution (1X PBS-Tween 0.05%+ 1% skimmed milk) added to each well. The plate was then washed again 3 times with PBS-Tween 0.05% and the primary antibody added. For the purpose of comparison, the primary antibodies used were typhoid sera, normal sera (from healthy blood donors) and an anti-GroEL monoclonal antibody (towards E.coli, StressGen, British Columbia). The plate was incubated with shaking for an hour, and then washed again with PBS-Tween 0.05%. Subsequently, 100μl of horseradish-peroxidase –labelled goat anti-human IgG conjugate (Pierce, Rockford, IL) was added to the wells that previously contained immune typhoid sera and normal sera and 100μl of horseradish-peroxidase –labelled goat anti-mouse conjugate (StressGen, British Columbia) added to the wells which previously contained

anti-GroEL monoclonal antibody. The plates were incubated for a further hour and washed again with PBS-Tween 0.05%. To conclude, 100µl of the chromogenic substrate (ABTS) with 0.01% H2O2 was added and the colour development observed. The optical density at 414nm was then read with an ELISA-plate reader (Titertek) after 40 minutes and the results tabulated.