CHAPTER 3

MATERIALS AND METHODS

3.1. Prawn collection

Prawn *M.rosenbergii* samples were collected from N.Sembilan , Malaysia. The infected individuals 16 brood stock (average body weight 15±2 g) transported using oxygenated water to the Monash Medical Research laboratory, Kuala Lumpur, Malaysia.

3.2. Serum Collection

Serum was collected from heart using 2ml disposable syringe and centrifuged at 12000rpm at 4ºC for 15min. The supernatant (about 50-100 µl) collected and stored at -80°C for further investigation.

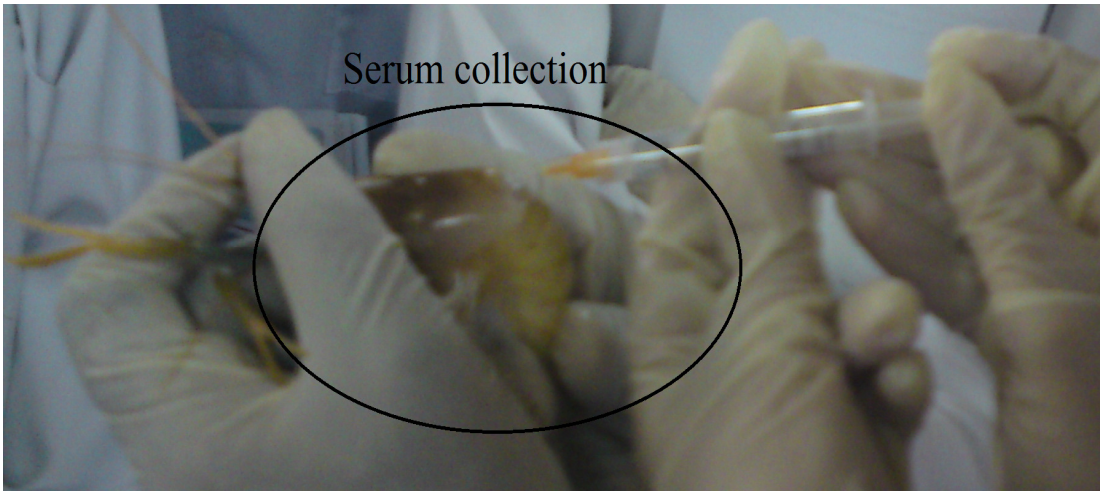

Figure 3.1, Serum Collection by cardiac-puncture technique, using 2ml syringe.
(Picture is taken by candidate at Monash University medical laboratory, Sunway campus)

3.3. Screening for IHHNV

Virus screening experiment was conducted to diagnose (IHHNV) infected prawns. Briefly, DNA was extracted from swimming leg using NucleoSpin Tissue, DNA extraction kite. A Nested PCR technique was applied to identify infected prawn using specific primer no 389. The primer sequence used for PCR detection is shown in table (Table 3.1).

Table 3.1, The primer sequence used for virus detection

	Designation Sequence Expected	Nucleotide start position	Amplicon size
IHHN V309F	5'-TCCAACACTTAGTCAAAACCAA3'	1936	309
IHHN V309R	5'-TGTCTGCTACGATGATTATCCA-3'	2244	

Crude IHHNV virus was obtained from IHHNV infected *Macrobrachium rosenbergii* prawn and it was used throughout this experimental work as positive conformation and Pleopods which are the abdominal appendages are often used for swimming, and hence are termed pleopods (Figure 3.2), 'swimming legs' or swimmerets used as the viral suspected DNA source.

Figure 3.2, Pleopods are located at abdominal appendage are cut to use for DNA extraction for Viral disease diagnosis.
(Picture is taken by candidate at Monash University medical laboratory, Sunway campus)

The first step PCR reaction mixture was prepared as 5µl of 5X buffer, 2 µl of 25mM $MgCl_2$, 2 µl dNTPs,2 Primer F 389,2 µl of primer R 389 0.25 µl DNA polymerase, 1 µl DNA template and top-upped with 13.75 µl dH_2O to final volume of 25 µl. After preparing the PCR mixture, the tube was placed in a PCR machine. A program was created based on the protocol from the manufacturer (Promega). It started with incubating the reaction mixture at 95º for 10 minutes. Then, followed by 95ºC for 2 minutes and decreased temperature to 55ºC for 5 minutes then increased the temperature 72 °C 1min (15 cycles) then continued the same condition for 5 min to disassociate the complementary DNA and RNA template. Finally, it was held at 4°C for 5 minutes.

In second step (Nested PCR (Promega) for IHHNV Detection) mixture was 5µl of 5X buffer, 1 µl of 25mM Mgcl2 , 2 µl dNTPs,0.5 Primer F 389,0.5 µl of primer R 389 0.25 µl DNA polymerase, 1 µl DNA template and top up with 14.75 µl dH_2O made the final volume of 25 µl. After preparing the PCR mixture, the tube was placed in a PCR machine. A program was created based on the protocol from the manufacturer (Promega). It started with incubating the reaction mixture at 95º for 2 minutes. Then, followed by 95ºC for 30 second and decreased temperature to 55ºC for 30 second then increased the temperature

72ºc (30 cycles) then continued with same condition for 5 min to disassociate the complementary DNA and RNA template. Finally, it was held at 4°C for 5 minutes.

3.4. Protein Determination of Serum

Quantitative estimation of serum protein was conducted using the dye binding technique of Bradford (1976) with modifications. The Bradford reagent was prepared by dissolving l00mg Coomassie Brilliant Blue G in 50ml of 95% ethanol. 100ml of 85 %(w/v) phosphoric acid was then added to the mixture. The mixture was diluted to a final volume of 1 litre with deionised water. The mixture was then stirred overnight followed by filtration and stored in a dark bottle. 0. lmg/ml of Bovine Serum Albumin (BSA) was prepared and used for protein standard curve. In this study, micro Bradford assays was used where the content of BSA contains 0 to 10u.g of protein. 1ml of Bradford reagent was added to loci of each crude serum extract (mg/ml) and the 6 standard solutions. The mixture was then incubated at room temperature for 20 minutes. The absorbance of the protein was measured at 595nm by using Shimadzu UV-160 spectrophotometer. The protein content of the samples was determined from the standard curve.

Protein concentration was determined between 2000, and 4000 times dilution. The amount of the loaded protein is calculated using the following formula:

$$C_1V_1 = C_2V_2$$

3.5. Bradford Assay

There are four well known spectroscopic methods used to determine the protein concentration in a solution. They are the Bradford dye assay, the Smith copper assay. The bicinchoninic assay and the Lowry assay. These methods all measure a protein's intrinsic UV absorbance and generate a protein-dependent color change.

UV absorbance requires that a pure protein with a known extinction coefficient be used, in a solution free of interfering (UV absorbing) substances. The Lowry and copper/bicinchoninic assays are based on the reduction of Cu^{2+} to Cu^{1+} by amides. This makes the assays very accurate, however, they require the preparation of several reagent solutions which must be carefully measured and mixed during the assay. This is followed by lengthy, precisely timed incubations at closely controlled, elevated temperatures, and then immediate absorbance measurements of the unstable solutions.

Both assays can be affected by other substances frequently present in biochemical solutions, such as detergents, lipids, buffers and reducing agents. As a result the assays must also include a series of standard solutions, each with a different, known protein concentration, but otherwise having the same composition as the sample solutions.

Compared to the other three methods, the Bradford assay is much faster. It involves fewer mixing steps, does not require heating, and gives a more stable colorimetric response. But like the other assays, its response is prone to be influenced by non protein sources, particularly detergents, and becomes progressively more nonlinear at the high end of its useful protein concentration range. The response is also protein dependent, and varies with the composition of the protein. These limitations make protein standard solutions necessary.

The Bradford assay is based on the use of a dye, Coomassie Brilliant Blue G-250. The light absorbing properties of the dye are altered when bound to a protein. When the dye is prepared as an acidic solution (in 85% phosphoric acid), it absorbs light with a maximum wavelength of 465 nm. The addition of protein results in a shift of the dye's maximum absorption to 595 nm. As the protein concentration increases, the absorbance of light at 595 nm increases linearly. This increase can be measured by a spectrophotometer. Although the absorbance of Coomassie blue dye at 595 nm is proportional to the amount of protein bound, it is necessary to establish a correspondence between absorbance values and known amounts of protein.

This is done by preparing a series of protein standards, which are dilutions of a protein solution with a known concentration. Once the A595 of each standard has been measured, it will be possible to plot the A595 as a function of the known protein content of each standard. After measuring the A595 of the unknown sample, the standard curve can then be used to determine the amount of protein corresponding to the absorbance values measured.

3.5.1. **Preparation of Bovine Serum Albumin (BSA) Protein Assay Standards**

In order to measure and plot a standard curve of protein concentration against absorbance at 595 nm, a series of dilutions of the BSA protein standard stock solution must first be prepared. The easiest way to calculate the volume of protein stock solution required for each dilution is by using the $C_1V_1 = C_2V_2$. The value of C_1 is the concentration of the protein stock solution, V_1 is the volume of the stock solution required, C_2 is the concentration of the diluted sample, and V_2 is the volume of the diluted sample. The

concentration of the stock solution, C_1, is 100µg/ml, the concentration of the diluted sample is C_2, and the volume of the diluted sample is fixed at 200 µl. Therefore the volume of stock solution required would be:

$$V_1 = C_2 V_2 / C_1$$

The protein standards should be prepared in the same buffer as the samples to be assayed. A simple standard curve can be made using bovine serum albumin with concentrations of 0, 10, 20, 30, 40, 50 µg/ml for the microassay (extinction coefficient of BSA is 0.667).

3.5.2. **Bradford Reagent**

The Bradford reagent can be made by dissolving 100 mg of Coomassie Blue G-250 in 50 ml of 95% ethanol; 100 ml 85% (w/v) phosphoric acid must also be added to the solution. Finally distilled water should be added to the mixture to achieve a final total volume of 1 liter.

3.5.3. **Procedure**

Prepare a 10-fold dilution of a 1 mg/ml BSA sample by adding 100 µl of 1 mg/ml BSA to 900 µl of distilled water to make 100µg/ml BSA. Generate test samples for the reference cell, blank, BSA standards and the protein sample to be tested according to Table 3.2 in disposable cuvettes. Note that a dilution of the protein sample may be required for the resulting absorbance to fall within the linear range of the assay.

Incubate each sample at room temperature for 5 minutes. Measure the absorbance of each sample at 595 nm using a UV-visible spectrophotometer. Allow the instrument to warm up for at least 15 minutes prior to use. Plot the absorbance of each BSA standard as a function of its theoretical concentration. The plot should be linear. Determine the best fit of the data to a straight line in the form of the equation

$$“\ y = mx + b\ ”$$

Where y is the absorbance at 595 nm and x is the protein concentration.

Use this equation to calculate the concentration of the protein sample based on the measured absorbance. If the absorbance of the test sample is not within the absorbance range of the standards, the assay must be repeated with a more appropriate dilution. The linear range for the assay and most spectrophotometers is 0.2 - 0.8 O.D. units.

Table 3.2, Preparation of Test Samples for the Bradford Protein Assay

Test Sample	**Sample Vol. (µl)**	**Water (µl)**	**Bradford Reagent (µl)**
Blank	0	200	800
BSA Standard (10 µg/ml)	20	180	800
BSA Standard (20 µg/ml)	40	160	800
BSA Standard (30 µg/ml)	60	140	800
BSA Standard (40 µg/ml)	80	120	800
BSA Standard (50 µg/ml)	100	100	800
Protein Sample	100	100	800

3.6. Two Dimensional Electrophoresis (2-DE) of Serum after Optimization

Briefly, the denatured serum (900 µ.g protein for coomasie blue stain; 450 µ.g protein for silver staining) were separated by isoelectric focusing in 13cm Immobiline dry strips(pH4-7; GE Healthcare Immobiline ^{TM} Drystrip). Then, proteins were separated in the second dimension by 15% SDS-PAGE and visualized after silver or coomasie staining.

3.7. First Dimension: Isoelectric Focusing

IPG (Immobilized Dry Strip) focusing was performed in individual strips containing immobilized pH gradients: IPG Dry Strips. These strips were rehydrated to their original thickness of 0.5rnm before use.

3.7.1. **Rehydration of IPG Dry Strips: Step 1**

The rehydration solution was prepared as mentioned in appendix 9. Briefly, volume of 250µl of rehydration buffer was pipeted into the 13cm Immobiline Drystrip Reswelling tray. The solution was delivered slowly at a central point of the tray. Large bubbles were removed. Immobiline Drystrip pH 4-7, 13cm was placed with the gel side down in the rehydration solution. Approximately 3ml of Immobiline Drystrip cover fluid was overlaid evenly across the surface of the IPG strip. The strips were allowed to rehydrate overnight for a period of at least 12-16 hours (Figure 3.3).

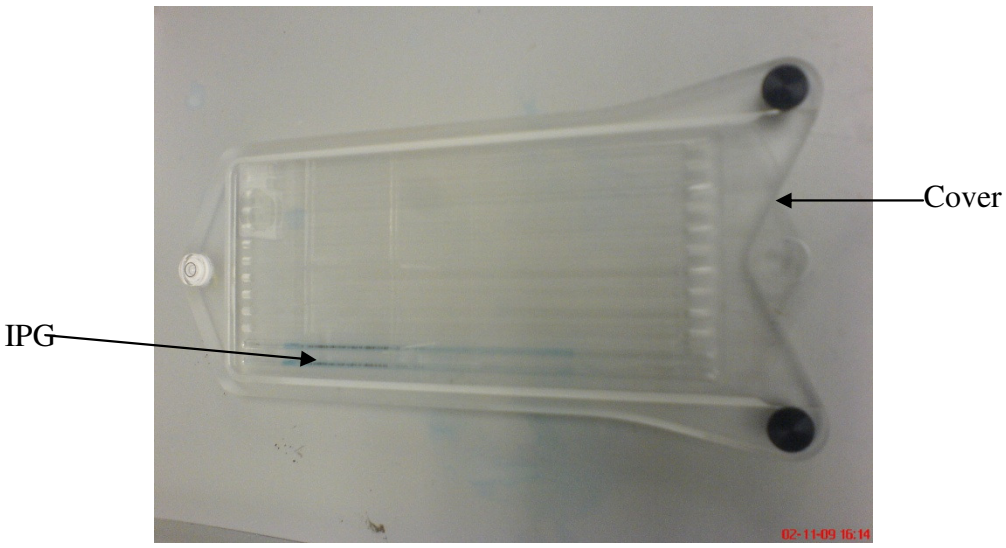

Figure 3.3, Dry strips are placed upside down in reswelling tray and covered by transparent cover for dry strip Rehydration
(Picture is taken by candidate at Monash University medical laboratory, Sunway campus)

3.7.2. **Rehydration: Step 2**

The Cup Loading Strip was positioned on the IPGphor platform (Figure 3.4). The rehydrated strips were then transferred to the holder. The IPG strip was placed face up in the tray with the anodic (+, pointed) end of the IPG strip toward the pointed end of the strip holder' Protrusions along the sides were used to guide the strip approximately Immobiline Dry-strip cover fluid was override evenly across the surface of the strip. Filter paper (BioRad Pads) was cut into 5mm square. Two pads per strip holder were required. The cut paper pads were dipped in deionized water and borated almost dry. The pads were placed on the gel of both ends of the strip. Next, an electrode was slid down on top of each cut paper pads. The sample loading cup was placed as close as possible to the anodic site of the strip by pressing the cup down until fully seated against the bottom of the strip holder. The strip is now ready for the loading of the sample.

Figure 3.4, IPGphor III Was used for protein Iso Electeric Focousing (IEF) on dry strip gels
(Pictures are taken by candidate at Monash University medical laboratory, Sunway campus)

3.7.3. **Sample preparation and running of lpGphor**

Depending on the requirement of each experiment the amount of protein was reconstituted accordingly to the calculated serum of rehydration solution. 800µg protein for coomasie blue staining and 450µg protein for silver staining method was prepared (Figure 3.5).

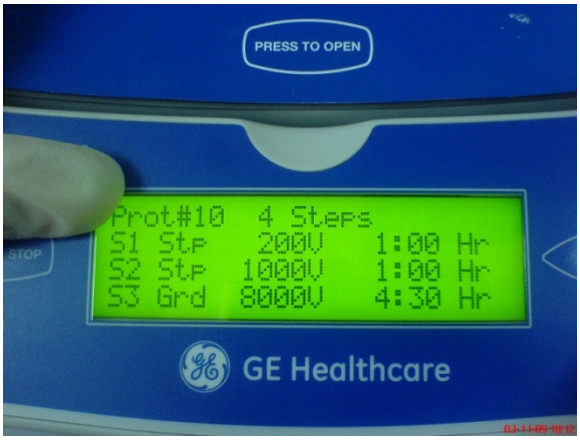

Figure 3.5, IPGphorIII programming Running condition was optimizing as 4 steps for 7h and 30 min under different voltage. Voltage is increasing gradually.
(Pictures are taken by candidate at Monash University medical laboratory, Sunway campus)

3.7.4. **Isoelectric Focusing (IEF)**

The strip might be run on the second dimension right away, or they were stored at -80^{o}C in a deep freezer (Figure 3.6).

Figure 3.6, IPGphor III, Ready for running 1D- Electrophoresis
(Picture is taken by candidate at Monash University medical laboratory, Sunway campus)

3.8. Second Dimension: SDS PAGE

The prerequisites to run second dimension requires the preparation of the following:

a. Acrylamide stock (30.8%), (appendix 1)

b. 1.5M Tris-Cl, pH 8.8, (appendix 2)

c. 10 %(w/v) SDS, (appendix 3)

The content of the above solution has been described in detail at the appendix section.

3.8.1. **Casting Homogenous Gel**

For the purpose of casting, the 2 gel casters were used. A total volume 100m1 of acrylamide solution was prepared for 2, 13 * 13.5 cm slab gels of 1.0mm thickness. The acrylarnide solution for 12.5% homogenous gels was prepared as follows (Table 3.3)

Table 3.3, 12.5% Homogenous gel solution

	Quantity (ml)
Acrylamide stock (30.8%)	41.7
1.5M Tric-CL, pH 8.8	25
Water	31.8
10%SDS	1
10%APS*	500µl
10%TEMED*	50µl

** The amounts of TEMED and APS may vary because of differences in temperature and reagent quality.*

The appropriate volume of APS and TEMED was added and stirred only when ready to pour the gel solution into the caster, Assembly of the gel caster to cast gels was as given in GE health Electrophoresis System user manual. The homogenous gels were allowed to polymerize for l hours before disassembling the caster.

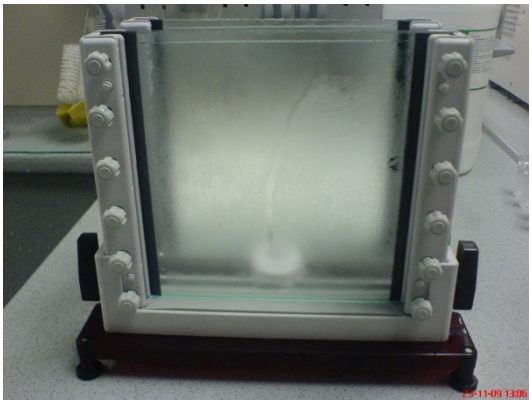

Figure 3.7, 2D-Gel casting was done at least 3h before running and kept it 4°C for at least 2h.

(Picture is taken by candidate at Monash University medical laboratory, Sunway campus)

3.8.2. **Preparing the Separation Unit**

The second-dimension electrophoresis or sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using the Ruby GE- Health Care System. The System requires a total volume of about 6L for each run to fill both the upper and lower chambers. Each run was performed by using two 12.5% homogenous gels.

3.8.3. **Preparing Second-Dimension Gels**

Equilibration and loading the first dimension ran strips were each equilibrated with 10ml of SDS equilibration buffer that has been added with concentration of 1% (w/v) DTT. The tubes containing the strips and equilibration solution were then incubated for 10min with gentle agitation. Following this, similarly as above a second equilibration of the strips was performed by preparing SDS equilibration buffer with addition of 2.5% (w/v) of iodoacetamide. During the equilibration, the gel cassettes were prepared for loading by rinsing the top of the gel with deionised water and draining. On completion of the equilibration, by using forceps, the equilibrated IPG strip were removed from the equilibration solution and rinsed with fresh SDS electrophoresis buffer. Before loading the equilibrated strips, precautions were taken by making sure that the gel surface and plates were dry. By holding one end of the IPG strip with forceps, it was drawn carefully across the long gel plate until the strip was completely on the glass plate and centered. Normally, the acidic end of the IPG strip was positioned on the left. Care was taken to avoid the gel face of the strip from touching the opposite glass plate. Using a thin plastic spacer it was then pushed to contact with the surface of the slab gel.

Low molecular weight marker was applied to a sample application piece made by cutting a square from filter paper (Whatman No.l). As described by the manufacturer, for coomasie blue staining the content of the vial was reconstituted in 200p1 of a standard of I x sample buffer (0.0625M Tris-HCL, 2% SDS, 0.lM DTT and 0.01% bromophenol blue, pH 6.8). From here 8pl of the solution was loaded onto the cut square of filter paper.

The IPG strip as well as the low molecular weight marker (if any) was then sealed in place by melting the agarose sealing solution in a microwave oven. The slightly cool

agarose solution was slowly pipetted across the length of the IPG strip, taking care not to introduce any large air bubbles. The agarose was allowed to cool and solidify for a minimum of 1 min. The gels were slid one by one into the electrophoresis tank once it reached the desired temperature. The Ruby System was then programmed to run for two gels as shown below:

3.9. Running condition

Running condition is dependeds on the gel concentration and the number of the running gel at the same time. Constant power is normally 20ºC. Programe attached in Appendix 12.

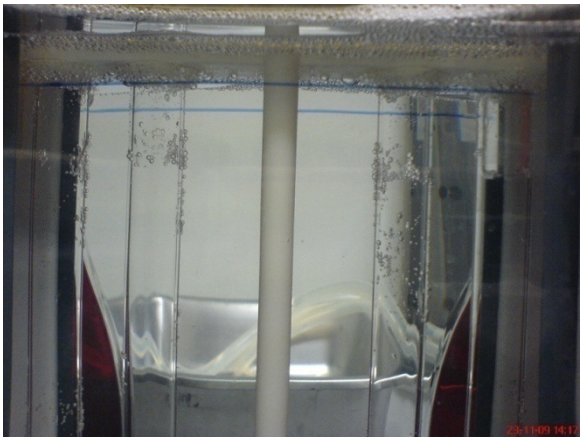

Figure 3.8, Completion of P1
(Picture is taken by candidate at Monash University medical laboratory, Sunway campus)

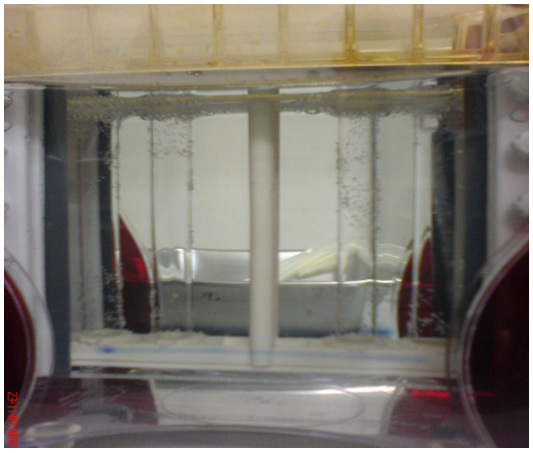

Figure 3.9, Completion of P2

(Picture is taken by candidate at Monash University medical laboratory, Sunway campus)

3.10. Detection by Staining

The acrylamaide gels were stained and visualized with both silver staining and coomassie blue technique. The techniques were carried as shown below:

3.10.1. **Fast Alcohol-Free Coomassie Staining**

To prepare staining solution one PhastGel Blue tablet (Coomasie R-350) dissolved in l.6L of l0% acetic acid. The solution was filtered (Whatman No.1) and stored in an opaque container. Prior to staining, 400m1 of the staining solution was heated to 90-100C in a water bath. The hot solution was poured into the gel in a stainless steel tray to stain for l5min on a shaker. The solution can be reused several times.

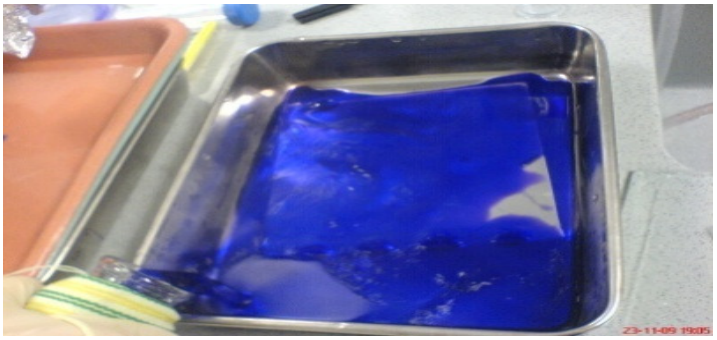

Figure 3.10, Coomassie Blue staining the background of the gel was distained by changing the l0% acetic acid distain solution several times for at least 5 hours at room temperature on 50 rpm shaker. The distained solution would be changed every 30 minutes
(Picture is taken by candidate at Monash University medical laboratory, Sunway campus)

3.10.2. **Silver Staining**

Silver nitrate technique was used in this study for silver staining method of Heukeshowven and Dernick (1985) (Heukeshoven and Dernick, 1985)with slight modifications. The detail protocol is presented in appendix-13.

Briefly, once electrophoresis was complete, gels were removed from glass plate and transferred into a container filled with fixing solution and were fully immersed in solution. Container was place on a shaker for 2 hrs or overnight. After 2 hrs, the fixing solution was thrown out and distilled water was replaced. Container was leave on a shaker at a speed of 80 rpm for 20 minutes. Washing step was repeated another 2 times. Distilled water was then removed and replaced by 20% ethanol for 20 minutes. The ethanol was then removed by pouring it out and washing the gel for 1 min with distilled water and sensitizing solution was added and the container was placed on the shaker for 20 minutes. This step was repeated another 2 times. The dH_2O was discarded, cold staining solution was added and container was placed on the shaker for 20 min. Subsequently, the staining solution was discarded and was washed with dH_2O for 30 sec; this step was repeated 2 times then, little

developing solution was added to rinse the gel and gel was coated with solution. Then gel container was topped up with developing solution and shacked by hand for approximately 3 to 5 min or until estimated spots or bands were visible. Following that, without discarding the developing solution, terminating solutions were added and the container was left on the bench for a short period, approximately 10 minutes to terminate the reaction. Finally, all solutions were discarded and gel was immersed in 1% acetic acid overnight.

3.11. Software Analysis

PDQuest Software was used for 2-D Gel Analysis and differential expression spots were selected for sequencing. (BioRad, Malaysia)

3.12. MALDI-TOF MS Analysis

MALDI-TOF MS is a method to demonstrate the utility of the peptides for enhanced analysis of proteins. In this method proteins were denatured and digested with trypsin. Trypsin is commonly used for protein digestion to produce peptides with molecular masses in the optimal range for MS analysis.

In proteomics research, tryptic peptides containing a C-terminal arginine throughout special ionization and are therefore more efficiently detected in MALDI-TOF MS when compared to lysine containing sp.

To reduce this preconception and increase overall ionization, lysine residues can be guanidinated to convert the -amine side chain to a homoarginine group as shown in Figure 22. Subsequent to guanidination, increased MS peak intensity is practical for lysine

containing peptides. This result in improves the ability to identify proteins by providing a larger number of candidates for peptide mass fingerprinting (PMF).

Regularly, the guanidination method is in use on solutions straightforwardly after tryptic digestion. guanidination was also performed on samples after spotting on a MALDI target and analysis.

3.12.1. **in-Gel Digestion**

In This study all samples were send to 1st base company to do protein spot identification. The method is described as below:

Before tryptic digestion, spots stained with Coomasie Blue were existed manually from the gels. They were subsequently stored at -20 ºC. The protocol followed was very similar to that published by Shevchenko (1996), albeit with a few modifications. 50pl of acetonitrile was added to the gel pieces and they were left for 15 minutes. This portion is known as dehydration where the gel pieces will shrink and may become opaque.

Once the supernatant was removed, the spots were rehydrated by adding 25 pl of 25 mM ammonium bicarbonate (NH_4HCO_3) and being left for 10 minutes.

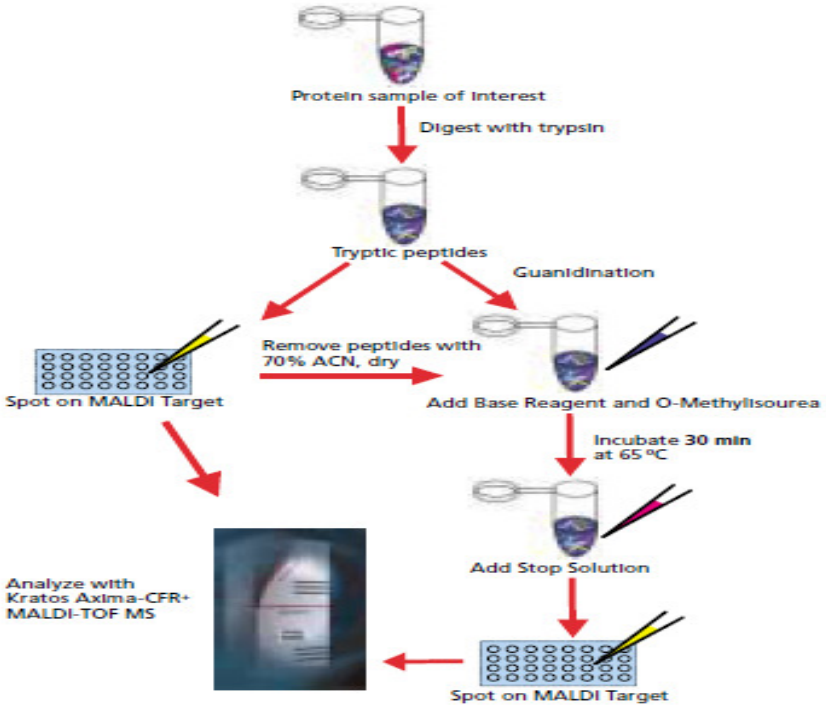

Figure 3.11, MALDI-TOF MS Analysis procedure (Steven Cockrill *et al.*, 2010)