Chapter Three
Materials and Methods

3.1 Materials

Materials used throughout this research are as follows:

3.1.1 *Cucumber mosaic virus*

This virus was kindly provided by Dr. Mohammad Roff from MARDI.

3.1.2 Single chain variable fragment (scFv) clone

The pET30a-scFv clone was a gift from Dr. J. Harikrishna (UM).

3.1.3 Tobacco Plants and Transgenic Tobacco Plants

Tobacco (*Nicotiana tabacum*) cv. White Burley plants were a gift from Dr. M.Roff from MARDI while the transgenic tobacco plants were obtained from Ng Cheah Wei (Genetic Molecular Laboratory,University of Malaya).

3.1.4 Specific Commercial Kits Used

- His Gravitrap (GE Amersham Biosciences)
- Reagent Set for Double Antibody Sandwich (DAS) ELISA (AGDIA, USA)

3.1.5 Instruments and Apparatus required

All instruments and apparatus used throughout this research are listed in Appendix A.

3.1.6 General Chemicals and Enzymes

The chemicals used were of analytical grade or the highest grade available. The list of chemicals and their respective manufacturers are in Table 3.1

Enzymes used were purchased from New England Biolabs®, USA, Qiagen, USA and Vivantis, Malaysia.

Table 3.1 : List of general chemicals used throughout this research and its respective manufacturers.

Chemicals	**Manufacturers**
B-mercaptoethanol	Sigma, USA
Acrylamide/bis solution	Bio-Rad, USA
Alkaline Phosphatase anti-mouse IgG	Sigma, USA
Ammonium acetate	BDH, ENGLAND
Ammonium persulfate	Bio-Rad, USA
Ampicilin disodium salt	Sigma, USA
Bovine Serum Albumin (BSA) Fraction V	Sigma, USA
Bromophenol Blue	Sigma, USA
Carborumdum	Fisher, UK
Chloroform	Merck, USA
Commassie R250	Sigma, USA
Dipotassium hydrogen phosphate	BDH, ENGLAND
Disodium hydrogen phosphate	BDH, ENGLAND
DTT	Sigma, USA
EDTA	Sigma, USA
Ethanol	Univar, USA
Glycerol	BDH, ENGLAND
Glycine	USB, USA
Glacial Acetic Acid	Univar, USA
Hydrochloric acid	BDH, ENGLAND
IPTG	Sigma, USA
Isoamyl alcohol	Univar, USA
Isopropanol	Univar, USA
LB agar	Pronadisa, Spain
LB broth	Pronadisa, Spain
Magnesium chloride	Sigma, USA
Methanol	BDH, ENGLAND
PBS tablet	Vivantis, MALAYSIA
PEG 8000	Sigma, USA
Phenol	Sigma, USA
PMSF	Sigma, USA
p-Nitrophenyl phosphate	Agdia, USA
Potassium chloride	Sigma, USA
Potassium dihydrogen phosphate	BDH, ENGLAND
PVP 40	Sigma, USA
SDS	Sigma, USA
Sodium acetate	Sigma, USA
Sodium azide	Sigma, USA
Sodium borate	ICN, USA
Sodium carbonate	Sigma, USA
Sodium chloride	Merck, USA
Sodium citrate	Sigma, USA
Sodium dihydrogen phosphate	BDH, ENGLAND
Sodium hydroxide	Sigma, USA
Sucrose	Sigma, USA
Thioglycolic acid	Sigma, USA
Tris	Promega, USA
Tris-HCL	ICN, USA
Triton X-100	BDH, ENGLAND
Tween-20	Sigma, USA

3.2 Methods

3.2.1 Cultivation of Tobacco Plants

All materials used for planting and growing of the plants were bought from commercial nurseries. Apparatus used were cleaned with 10% clorox solution and rinsed with distilled water, while materials were sterilized by autoclaving prior usage.

The soil was made up of 100% humus-rich multi-purpose soil autoclaved at 121 psi for 15 minutes. The planting pots, watering pots and other gardening implements were cleansed with 10% clorox solution and rinsed with distilled water. Holes at the bottom of the planting pots were covered with autoclaved paper to avoid soil leakage.

Tobacco (*Nicotiana tabacum*) seeds were immersed in distilled water to verify their viability by the appearance of sunken seeds after a few minutes. Viable seeds were implanted into loosely-moistened soil 5 inches apart for germination, then transplanted into single pots (one plant per pot). Healthy 14-day old plantlets with first three leaves developed were used for inoculation.

3.2.1.1 Growth condition for Tobacco plants

Tobacco plants were kept in an environmentally controlled growth chamber (Conviron, Canada) with programmable temperature and light intensity levels. The plants were maintained at a cycle of 16 hours of light (25℃) and 8 hours of dark (16℃).

3.2.2 Inoculum and Infection Studies

3.2.2.1 Inoculation of Virus

Young CMV-infected leaves of cucumber obtained from Dr. M. Roff (MARDI) were macerated in pre-chilled PBS buffer, pH 7.5 (Appendix B) containing autoclaved

carborundum (Fisher, USA) in a pre-chiled mortar and pestle. The crude sap was mechanically innoculated onto tobacco plants by gently rubbing the surface of tobacco young leaves or cotyledons with gloved hands. For mock-inoculation, distilled water was used in place of CMV on the leaves as well. Excess inoculum on the leaves was rinsed away with distilled water after 15 minutes post-inoculation.

Healthy CMV-free tobacco plants were grown separately in another environmentally controlled growth chamber under the same conditions.

3.2.2.2 Monitoring of Infected Tobacco Plants

Appearance of symptoms were observed and monitored on the infected plants. Symptoms such as necrotic local lesions, systemic mosaic, green blisters and leaf deformations appeared approximately 14 days post-inoculation (d.p.i).

3.2.3 Isolation and purification of *Cucumber mosaic virus*

Purification of the virus was done according to the procedure of *Lot et al* (1972). The virus isolate was propagated in *N. tabacum* plants by mechanical inoculation. Fresh infected leaves (20 g) were harvested two weeks after virus inoculation (14 d.p.i) and weighed, after removing major ribs and stems. The leaf tissues were homogenized in a pre-chilled blender, in buffer A (Appendix C) and cold chloroform, at a ratio of 1:1 (w/v). The homogenate was transferred to a 250ml centrifuge tube and centrifuged at 15,000g at 4℃ for 10 min. The aqueous phase was filtered through a dampened Miracloth, and the filtrate was transferred into 25ml ultracentrifuge tubes.

Prior to the first ultracentrifugation step, the virus was concentrated by precipitation with polyethylene glycol (PEG). PEG 8000 was added into the filtrate at a ratio of 10 : 90 (filtrate weight in g : buffer A volume in ml) The mixture was stired

at 4℃ for 45 min. After the PEG had dissolved, the mixture was centrifugated at 15,000g at 4℃ for 10 minutes. The supernatant was discarded and the pellet was then drained thoroughly. The pellet was resuspended in buffer B (Appendix C), using one-third of the original extraction volume, and stired for 45 minutes at 4℃. After the virus pellet had dissolved completely, the mixture was centrifugated at 7500g at 4℃ for 10 minutes. By then, the supernatant containing the virus was collected and kept on ice. Any remaining pellet was resuspended in buffer B and kept at -20℃.

A Beckman L-80 ultracentrifuge machine with a 80ti rotor was vacummed to 4℃. The 13ml ultracentrifuge tubes were firstly filled with 5ml of cushion II solution (Appendix C) using a sterile needle and syringe. By tilting the tube 45°, the cushion II solution was carefully added to avoid bubble formation. Any remaining drops of cushion II along the wall was eliminated by tilting the tube so that the edge of cushion II touched the remaining drops. Any bubbles formed was removed by gently tapping the tube wall. Again, by tilting the tube 30° horizontally, the virus supernatant was gently flowed along the tube wall without disturbing cushion II until the tube was fully filled. The volume of supernatant was topped up with buffer B as required, and any remaining supernatant was kept at -20℃. The ultracentrifuge tubes were balanced and hot-sealed. The ultracentrifuge tubes were centrifugated at 40,000 rpm for 1.5 hour at 4℃.

Next, the supernatant was discarded, and 2-5ml of buffer B (Appendix C) was added to each pellet depending on the pellet size. The pellets were then resuspended at 4℃ overnight. After the pellets had dissolved completely, the samples were pooled and centrifuged at 7500g at 4℃ for 10 minutes. The supernatant containing the virus was collected, and the ultracentrifugation steps repeated.

The final virus pellet of virus was resuspended in buffer C (Appendix C) and stored at -20℃ in 50% glycerol. Quantification of the virus yield was done using a

Biophotometer (Eppendorf, USA) at OD_{260} with extinction coefficient of 5 (Francki *et al*,1966). Virus purity was further analyzed using SDS-PAGE.

3.2.4 Expression of scFv in *Escherichia coli* and Transgenic Tobacco plants

3.2.4.1 Bacteria Cell culture

E. coli BL21 (DE3) cells (Novagen, USA) transformed with pET30a-scFv were grown overnight at 30℃ with vigorous shaking at 250 rpm in 5ml of Luria-Bertani (LB) medium (Appendix B) containing 30μg/ml kanamycin and 34μg/ml chloramphenicol. The cells were then transferred to 100ml LB medium, supplemented with the same concentration of antibiotic as mentioned earlier, and grown with shaking to reach an OD_{600} of 0.6 (approximately 1.5 hour). To induce the scFv expression, one mM IPTG was added and the culture was incubated for 4 hours at 30℃.

3.2.4.2 Cultivation of Transgenic Tobacco Plants

The preparation for cultivation of transgenic plants was the same as described in Section 3.2.1. Transgenic tobacco (*Nicotiana tabacum*) seeds were immersed in distilled water to verify their viability by the appearance of sunken seeds. Viable seeds were planted in loosely-moistened soil 5 inches apart for germination. Healthy 14-day old plantlets with first three leaves were transplanted to individual pots.

3.2.4.3 Growth conditions for Tobacco plants

The transgenic tobacco plants were subjected to the same growth conditions as the wild type, as described in Section 3.2.1.1.

3.2.5 Extraction and Purification of scFv from *Escherichia coli* and Transgenic Tobacco plants.

All extraction and purification steps were carried out at 4 ℃.

3.2.5.1 Extraction of scFv from *Escherichia coli*

Cells were harvested by centrifugation at 12,000 rpm for 20 min and washed with PBS buffer (Appendix B). The cells were suspended in binding buffer, pH 7.4 (Appendix E) at a ratio of 1:5 (w/v) (g/ml). Cells were disrupted with 1mg/ml lysozyme and incubation for 30 minutes on ice. The cell lysate was then cleared by centrifugation at 14,000 rpm for 15 minutes. The supernatant was kept at -20 ℃ prior to further purification.

3.2.5.2 Extraction of scFv from Transgenic Tobacco plants

20g of leaf tissues was ground with liquid nitrogen using a mortar and pestle. The sample was then transferred into 250ml centrifuge tube and 200ml of extraction buffer (Appendix D) was added. The tube was vortexed for 30 seconds and 200ml of Tris-phenol, pH 7.5 (Appendix D) was added into the extract. Mixing was done by vortex for another 30 seconds. The extract was then centrifuged at 10,000g for 10 minutes at 4 ℃. The phenolic phase was collected and transferred into 250ml centrifuge tube and 200ml of extraction buffer was added again. Mixing was repeated with vortex, and the extract was centrifugated at 10,000g for 15 minutes at 4 ℃.

The phenolic phase was collected into 25ml centrifuge tube, and the proteins were precipitated overnight with 5 volume of 100mM Ammonium acetate in absolute methanol (Appendix D) at -20 ℃. The solution was then centrifuged for 30 minutes at 4 ℃. The supernatant was removed, and the pellet was drained thoroughly. Subsequently, the pellet was rinsed twice with ice-cold Acetone containing 0.2% DTT.

The pellet was left to air dry and resuspended in either 100 µl of protein resuspension buffer (Appendix D) for storage at -20 ℃ or 100 µl of binding buffer (Appendix E) with 2% (w/v) SDS for subsequent purification.

3.2.5.3 Small-scale purification of scFv from *Escherichia coli* and Transgenic Tobacco plants

His Gravitrap™ column (Amersham Biosciences) was pre-equilibrated with 10 ml of binding buffer (Appendix E). The extract/supernatant was loaded into the column with gravity flow. Once the extract had completely passed the bed volume, the column was then washed with 12ml of binding buffer (Appendix E). The bound proteins were eluted off the column with 2.5ml of elution buffer (Appendix E). Eluted proteins were pooled together and dialyzed twice in 500ml of binding buffer for 6 hours. The proteins were concentrated by dialysis with 500ml of binding buffer in 50% glycerol for 30 min. The concentrated proteins were again loaded into the His Gravitrap™ column for further purification. All purification steps were repeated. The purified proteins were stored at -20 ℃ with 20% glycerol and were nearly pure, as assessed by SDS-PAGE.

3.2.6 Screening of CMV and scFv

3.2.6.1 Sodium Dodecyl Sulfate PolyAcrylamide Gel (SDS-PAGE) and Electrophoresis

Discontinuous 12% SDS-PAGE gels (1 mm thick, 10 wells) were prepared using Mini-PROTEAN® Cell (Bio-Rad, USA) according to the manufacturer's protocols.

Protein/samples (15ul) were denatured under reducing conditions by the addition of 5ul 3X sample protein buffer (Appendix F), followed by boiling of the mixture for 3 minutes in a dry bath and lastly a quick chilling on ice for at least 1 minute. Mixture in tubes were suspended and short-spinned prior to loading onto the gel.

Following the assembly of Mini-PROTEAN® Cell with a freshly-casted gel and addition of 1X running buffer (Appendix F), the mixtures (20ul) were loaded into the sample wells with the aid of gel-loading tips. Either Perfect Ladder protein marker (Vivantis, Malaysia) or Prestained Protein Ladder (Fermentas, Canada) was also loaded into a well for comparison with samples or subsequent Western transfer of proteins.

Electrophoresis was carried out at 180 V and terminated when the dye front reached to the bottom of the gel. Typical run times were approximately 57 minutes.

3.2.6.2 Staining

Immediately following electrophoresis, the gel was immersed into fixing buffer (Appendix F) and stained with Commassie Blue Staining Solution (Appendix F). The gel was incubated with shaking on a belly dancer for 2 hours. The staining solution was then discarded and replaced with destain solution I (Appendix F). The gel was shaken again for 30 minutes to remove excess stains. Destain Solution I was discarded, and destain solution II (Appendix F) was used and changed periodically until the gel background was clear. The gel was viewed using a light box or recorded using gel documentation system (AlphaImager 2000).

Silver Staining : After electrophoresis, the gel was immersed into fixing solution (Appendix F) for 30 seconds and followed by immersing it into infiltrating solution (Appendix F) for 30 seconds. Then, the solution was discarded and the gel was washed three times with distilled water for every five minutes. The gel then was

stained with silver solution (Appendix F) for 20 seconds. After removing the silver solution, washing was done twice with distilled water for every one minute. The gel was immersed into staining solution (Appendix F) between two to ten minutes, depending on the preferred specific band intensity. Immediately after that, the staining was stopped by transferring the gel into stop solution (Appendix F). Residual of the stop solution was removed by washing the gel three times with distilled water for every five minutes. The gel is best kept in conserving solution (Appendix F).

3.2.7 Serological Studies

The serological methods used in this study were Western Blot, Dot Blot and ELISA for detection of virus using the scFv antibodies.

3.2.7.1 Western Blot

Prior to the completion of SDS-PAGE, a sheet of Hybond-P PVDF membrane (Amersham Biosciences, USA) was cut to the gel size. The membrane was pre-wetted in absolute methanol for 10 seconds and rinsed with distilled water for 5 minutes. The membrane was then equilibrated in protein transfer buffer (Appendix G) for 10 minutes. After the separation of proteins by SDS-PAGE, the stacking gel was removed and the separating gel was orientated by cutting off a small corner. The gel was immersed in protein transfer buffer for a minute to equilibrate. The gel and membrane were sandwiched between a stack of filter paper which had been pre-wetted with protein transfer buffer.

The electroblotting casette for the Mini Transblot® Electrophoretic Transfer Cell (Bio-Rad, USA) was assembled and placed between the electrodes in the blotting unit, as in the manufacturer's instruction. Transfer was performed in cold protein transfer buffer for an hour at 100V with constant cooling. As soon as the transfer was completed, the membrane was removed from the electroblotting casette. Success of

transfer can be observed by the presence of the pre-stained protein ladder and the orientation of the gel on the membrane was marked by cutting a corner of the membrane. Briefly, the membrane was rinsed with TBS buffer (Appendix G). The membrane can either be used immediately or air dried prior to storage. The membrane can be kept between sheets of Hybond blotting paper, wrapped in SaranWrap at 4℃ up to 3 months. Pre-wetting the membrane is required as mentioned above prior usage.

3.2.7.2 Dot Blot

A sheet of Hybond-P PVDF membrane (Amersham Biosciences, USA) was cut out, sectioned and labelled with a pencil. The membrane was pre-wet as mentioned above. Protein samples were then applied onto the designated sections of the membrane. After applying the samples, the membrane was dried for a short time at room temperature before proceeding with the immunodetection process.

3.2.7.3 Immunodetection

Equilibration of the membrane was done by washing the membrane twice for 10 minutes each time in TBS buffer (Appendix G). After which was followed by immersing the membrane for non-specific binding in 5 % dried skimmed milk (w/v) added to TBS buffer (Appendix G) with incubation for an hour at room temperature. The membrane was then washed twice in excess volume of TBST (Appendix G) and subsequently washed once with TBS buffer, both with agitation for 10 minutes each time on a belly shaker. The membrane was incubated with a 1/2000 dilution of Penta-Anti.His IgG mouse antibody (Qiagen, USA) in 5% dried skimmed milk in TBS buffer (Appendix G) for one hour at room temperature.

The washing of the membrane was repeated as described above. The membrane was then incubated with Alkaline Phosphatase (AP) conjugated anti-mouse IgG in 5%

dried skimmed milk (w/v) in TBS buffer (Appendix G) for one hour at room temperature.

The membrane was washed 4 times with TBST, and stained with BCIP (Sigma, USA) solution (Appendix G) in darkness for colour development. As soon as the signal was clearly visible, the reaction was stopped by rinsing the membrane twice in distilled water. The membrane was then dried and photographed before storage. The protocol above was modified depending on the usage of appropriate antibodies and experimental requirements.

3.2.7.4 ELISA

Enzyme-linked Immunosorbent Assay (ELISA) was carried out using a reagent set (Agdia, USA) for Double Antibody Sandwich (DAS) ELISA. The procedures used were as in manufacturer's protocol:

The 96-well microtitre plate (Grenier, USA) was pre-coated with 100µl of anti-CMV capture antibody (Agdia, USA) using coating buffer (Appendix H) as in the manufacturer's protocol and incubated either overnight at 4℃ or 4 hours at room temperature. The microtitre plate was washed by emptying the wells into the sink and filled with overflowing of 1X PBST (Appendix H). Washing of wells were repeated 3 times, with each time the plate was placed upside down and firmly tapped on folded paper towel to remove excess liquid in the wells. Crude sap from young CMV-infected leaf samples were ground in General Extraction Buffer (GEB) (Appendix H) at a ratio of 1:10 (samples weight in g : buffer volume in ml) using a mortar and pestle. Based on a loading diagram (see Figure 3.1), 100µl of prepared samples were dispensed into designated sample wells. With the same volume, positive controls (Agdia, USA) were dispensed into positive control wells while negative controls (uninfected plant samples) were dispensed into negative control wells. The GEB was then loaded into buffer

wells. The plate was sealed using sealing film and incubated for 2 hours at room temperature.

Prior to the next step, the anti-CMV alkaline phosphatase enzyme conjugate was prepared at a ratio of 1: 200 (Agdia, USA) dilutions in ECI buffer (Appendix H) as secondary antibody, following the manufacturer's instruction. Washing of the plate was repeated 7 times as described previously. Inspection of each test wells was done to ensure absence of plant tissue. 100µl of prepared enzyme conjugate solution was dispensed into each well, and the plate was incubated for 2 hours at room temperature. Para nitrophenyl phosphate (PNP) tablet (Agdia, USA) was used as substrate for developing colour and diluted in PNP buffer (Appendix H) to a concentration of 1mg/ml. After washing the plate 8 times consecutively, any presence of air bubbles was removed by a clean pipette tip. Then, 100µl of PNP substrate was dispensed into all wells, and the plate was incubated in darkness for a duration of 30 to 60 minutes at room temperature. Finally, absorbance was measured by reading the plate at 405 nm, and reaction was then stopped using 3N NaOH.

Wells in which colour develops indicate positive results and those with no significant colour development indicate negative results. Test results are valid only if positive control wells give a positive result and negative as well as buffer wells remain colourless.

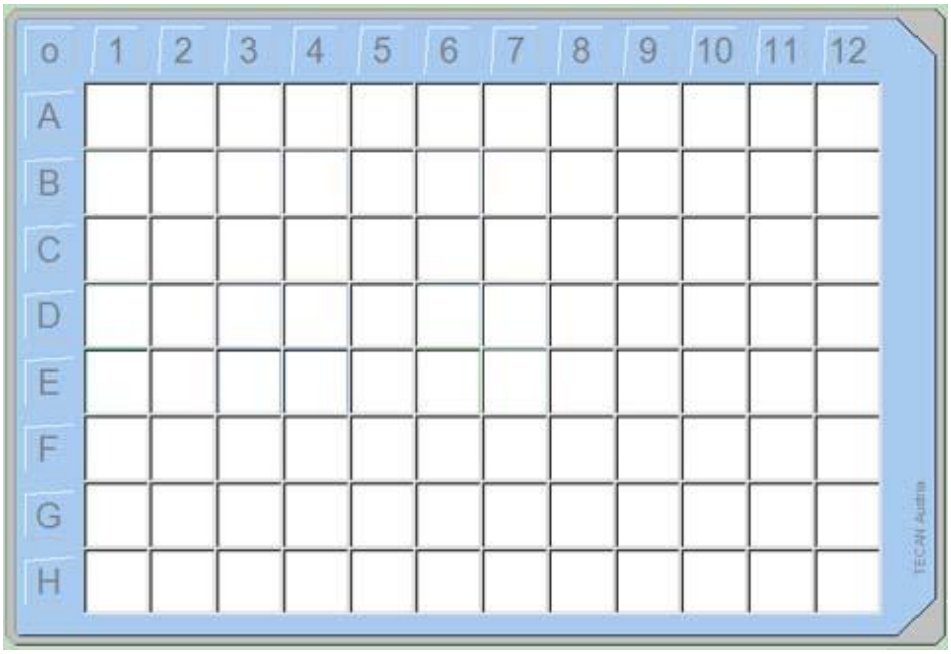

Figure 3.1 : ELISA loading diagram.

3.2.8 Checkerboard Titration ELISA

In this study Checkerboard titration DAS-ELISA was selected for developing an assay for the recombinant scFv. The scFv was used as the capture antibody with a starting dilution of 1/1 (see Table 3.2).

A 96-well microtitre plate (Grenier, USA) was pre-coated with coating buffer (Appendix H) from column 2 to column 12 (Table 3.2). One ml of scFv diluted in PBS buffer, pH 7.4 (Appendix H) was prepared, and 50µl of it was mixed into wells of column 1 and 2 using a multichannel pipetter (Eppendorf). The antibody was diluted across the plate from column 2 to column 11, by transfering and mixing between each column and discarding the last 50µl in column 11. By then, each well on the plate contained only 50µl of antibody-coating buffer, with column 1 as starting dilution of 1/1, followed by dilution range of twofold across the plate and column 12 filled only with coating buffer.

The plate was sealed with sealing film and incubated either overnight at 4℃ or 4 hours at room temperature. The microtitre plate was washed by emptying the wells into the sink and filled to overflowing of 1X PBST (Appendix H). Washing of wells were repeated 3 times, and after each wash the plate was placed upside down and firmly tapped on folded paper towel to remove excess liquid in the wells.

After the washing of the plate, 50µl of PBS buffer was dispensed into wells of row A to row H (Figure 3.2). Purified CMV antigen was diluted from row A to row G, loading 50µl of antigen into row A, transfering and mixing between each row and lastly discarding the last 50µl in row G. Thus, each well on the plate contained only 50µl of antigen-PBS buffer, with row A as starting dilution of 0.03 mg/ml, followed by dilution range of twofold across the plate and row H filled with only PBS buffer. The plate was sealed again and incubated for 2 hours at room temperature.

Prior to next step, the anti-CMV alkaline phosphatase enzyme conjugate was prepared at 1: 200 (Agdia, USA) dilutions in ECI buffer (Appendix H) as secondary antibody, following the manufacturer's instruction. Washing of the plate was repeated 7 times as described previously. Inspection of each test wells was done to ensure absence of air bubbles. 50 µl of prepared enzyme conjugate solution was dispensed into each well, and the plate was sealed and incubated for 2 hours at room temperature. Para nitrophenyl phosphate (PNP) tablets (Agdia, USA) were used as substrate for developing colour and diluted in PNP buffer (Appendix H) to a concentration of 1mg/ml. After washing the plate 8 times consecutively, any presence of air bubbles was removed by a clean pipette tip. Then, 100 µl of PNP substrate was dispensed into all wells, and the plate was incubated in darkness for a duration of 30 to 60 minutes at room temperature. Finally, absorbance was measured by reading the plate at 405 nm, and reaction was then stopped using 3N NaOH.

Table 3.2: Schematic Serial dilutions of scFv antibody and antigen in an ELISA plate.

	scFv Dilutions	1:1	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	Buffer
Antigen Dilutions	0	1	2	3	4	5	6	7	8	9	10	11	12
1: 50	A												
1:100	B												
1:200	C												
1:400	D												
1:800	E												
1:1600	F												
1:3200	G												
1:6400	H												

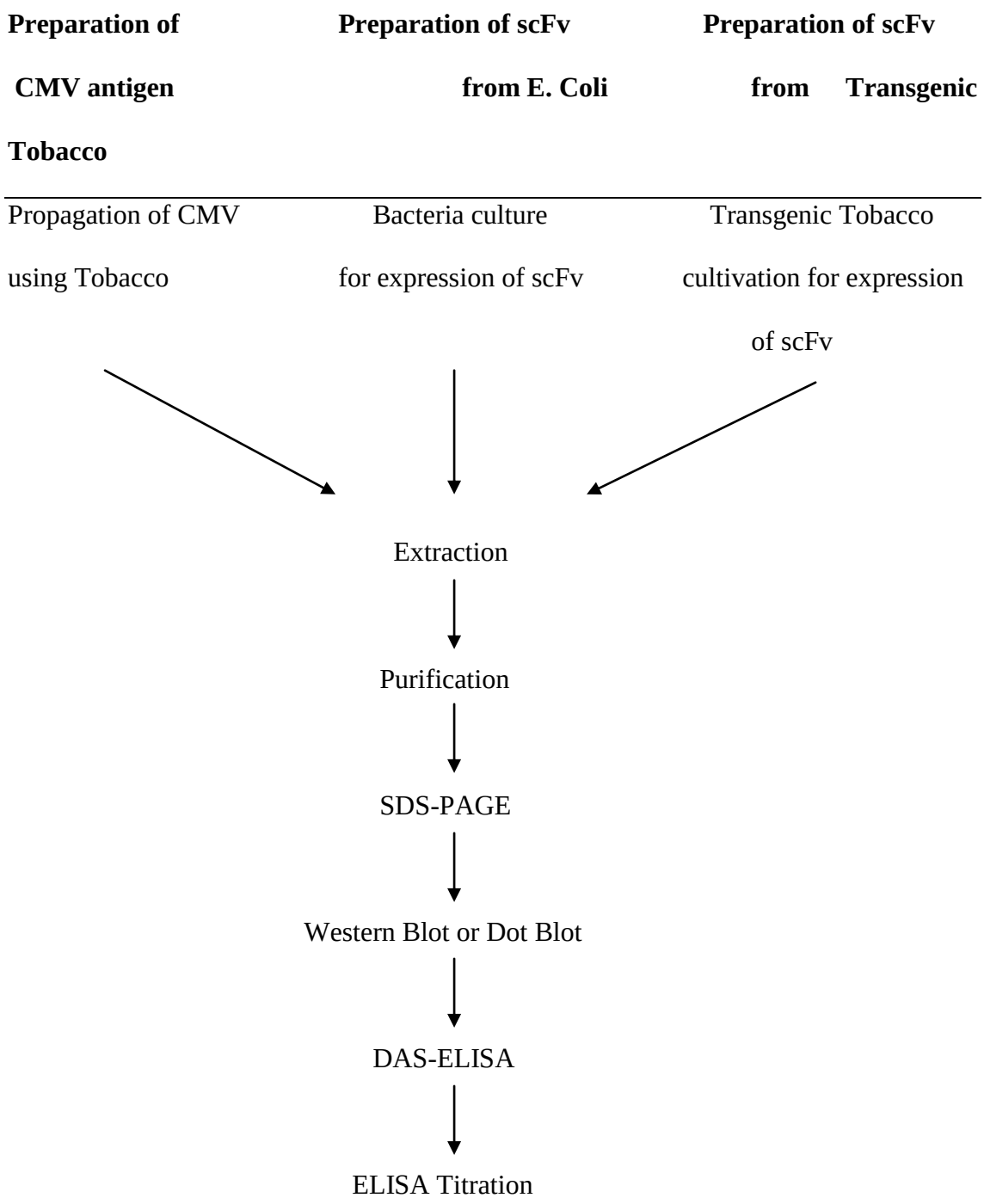

Figure 3.2 : Schematic summary of project workflow.