CHAPTER II

Materials and Methods

1. Construction of expression vector

In principle, DNA cloning is a technique for reproducing DNA fragments. A vector is required to carry the DNA fragment of interest into the host cell. Creating recombinant DNA, A plasmid vector is digested with restriction enzymes to produce two sticky ends and desired DNA insert is also digested with the same restriction enzymes to produce pieces with the compatible sticky ends. The two samples are mixed and allowed to hybridize and then some molecules will form with pieces of interested DNA inserted into the plasmid vector at the restriction enzyme sites. DNA ligase is used to covalently link the fragments. The recombinant vector must also contain an antibiotic-resistance gene. The recombinant DNA enters into the host cell and proliferates. It is called "transformation" because the function of the host cell may be altered. Normal *E. coli* cells are difficult to take up plasmid DNA from the medium. If they are treated with $CaCl_2$, the transformation efficiency can be significantly enhanced. Even so, only one cell in about 10000 cells may take up a plasmid DNA molecule. A specific antibiotic is added to kill *E. coli* without any protection. The transformed *E. coli* is protected by the antibiotic-resistance gene whose product can inactivate the specific antibiotic. The numbers of vectors in each *E. coli* cell are not the same, because they may also reproduce independently.

1.1. Preparation of p53 encoding DNA for insertion into pAK400 vector and pET-15b vector

The p53 encoding DNA was generated using PCR. The PCR process usually consists of a series of thirty-five cycles. Each cycle consists of three steps. Prior to the first cycle, the double-stranded DNA has to be heated to 95 °C for 5 minutes in order to separate the strands. This step is called denaturing; it breaks apart the hydrogen bonds that connect the two DNA strands. First the DNA is often denatured for an extended time to ensure that both the template DNA and the primers have completely separated and is now single-strand only. The temperature in this stage is 95 °C for 30 seconds. After separating the DNA strands, the temperature is lowered so the primers can attach themselves to the single DNA strands. This step is called annealing. The temperature of this stage is 50 °C for 30 seconds. Finally, the DNA polymerase has to copy the DNA strands. It starts at the annealed primer and works its way along the DNA strand. This step is called elongation. The elongation temperature is 72 °C for 2 minutes. A final elongation step is frequently used after the last cycle to ensure that any remaining single stranded DNA is completely copied. This differs from all other elongation steps, 7 minutes.

The p53 target gene in pcDNA3.1 (a kind gift from Dr. John Lunec, Northern Institute for Cancer Research, England) was amplified in a PCR reaction and the incorporation of appropriate restriction enzyme target sequences on the PCR primers are required for production of sticky-ended DNA molecules available for the insertion into vector.

NdeIp53, forward primer and p53EcoRI, reverse primer (Provided by Dr. Chatchai Tayapiwattana, Associated medical sciences, CMU) were used to generate

NdeI-p53-EcoRI PCR product (target DNA to be inserted into pAK400 vector) as, indicates in Figure 3.

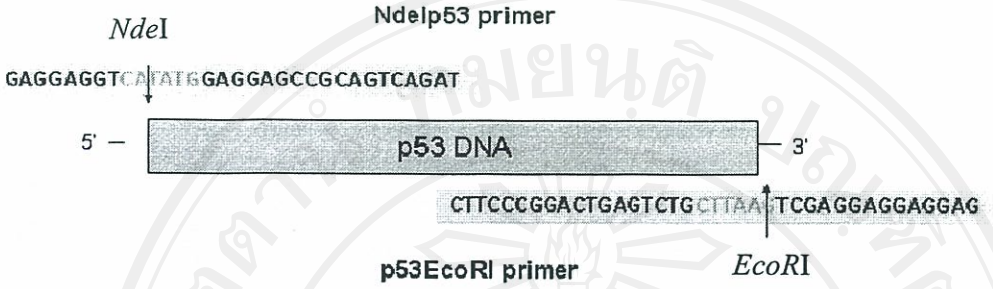

Figure 3. The p53 target gene in pcDNA3.1 was amplified by NdeIp53 primer and p53EcoRI primer to generate NdeI-p53-EcoRI PCR product

NdeIp53, Forward primer and p53BamHI, reverse primer were used to generate NdeI-p53-BamHI, target DNA to be inserted into pET-15b vector as, indicates in Figure 4.

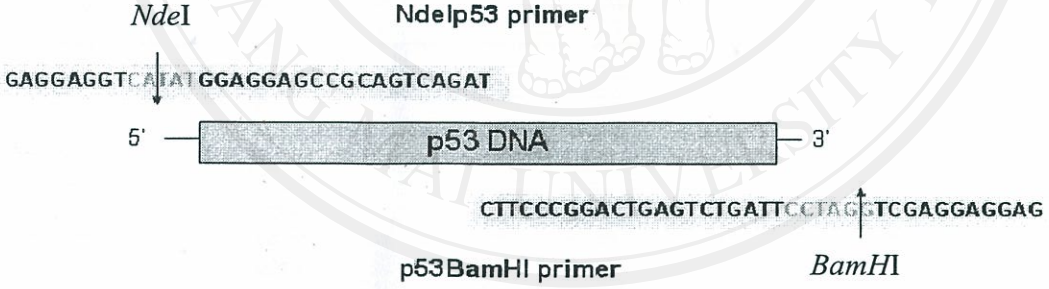

Figure 4. The p53 target gene in pcDNA3.1 was amplified by NdeIp53 primer and p53BamHI primer to generate NdeI-p53-BamHI PCR product

To reduce the number of potential mutations that may occur during PCR amplification, *proofstart* DNA polymerase (QIAGEN) was chosen for PCR amplification. The expected PCR products are around 1500 nucleotides in length. To

produce target DNA, the 50-µl reaction was performed with the mixture as described in Table 1.

Table1. PCR reaction mixture contents

Reagent	**Concentration**	**Volume(µl)**
Sterile distilled water		32.5
10x buffer		5.0
dNTP	50 mM each	5.0
Proofstart DNA polymerase	50 unit/µl	0.5
primer (forward)	0.5 µM	2.5
primer (reverse)	0.5 µM	2.5
Template	25 ng/µl	2.0

A 200 µl reaction tube containing the 50 µl mixture was then placed into the thermocycler (2231, eppendorf). Programmed as follows:

	95°C, 5 minutes	
Denaturation	95°C, 30 seconds	35 cycles
Primer annealing	50°C, 30 seconds	
Primer extention	72°C, 2 minutes	
	72°C, 7 minutes	

The PCR product can be identified by its size using agarose gel electrophoresis. Agarose gel electrophoresis is a procedure that consists of administrating 5 µl of PCR product mixed with loading buffer (Fermentas) to the ratio of 6:1 into agarose gel and then applying a constant 50 voltage electric current to the gel. As a result, the smaller

DNA strands move faster than the larger strands through the gel toward the positive current. The size of the PCR product can be determined by comparing its size with a 50 bps DNA ladder (Fermentas), which contains DNA fragments of known size range between 50-1031 bps, also included within the gel.

1.2 Cloning of p53 encoding DNA into pAK400 vector

1.2.1 Digestion of pAK400 vector and NdeI-p53-EcoRI PCR product with restriction enzymes

First, the pAK400 vector was double digested with *NdeI* and *EcoRI* to linearize the supercoiled plasmid. Meanwhile, NdeI-p53-EcoRI PCR product was also cut with *NdeI* and *EcoRI* to produce stricky ends available for the insertion into vector. For digestion of pAK400 vector, a 500 µl reaction tube containing the 20 µl mixtures consists of 80 ng of pAK400 vector, 10 units of *NdeI*, 10 units of *EcoRI* and 2 µl of R buffer. For digestion of NdeI-p53-EcoRI PCR product, a 500 µl reaction tube containing the 10 µl mixture consists of 120 ng of NdeI-p53-EcoRI PCR product, 10 units of *NdeI,* 10 unit of *EcoRI,* 1 µl of R buffer and 4 µl of sterile distilled water. Both mixtures were incubated into the water bath overnight at 37 °C. Prior to ligation, DNA insert was purified using QIAquick PCR purification kit (QIAGEN GmbH). Meanwhile the linearized plasmid DNA was gel purified using the QIAquick Gel extraction kit (QIAGEN GmbH).

QIAquick PCR Purification is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge. The method started with applying 5

volumes of buffer PB to 1 volume of the PCR sample and mixed thoroughly. Then sample was applied to the QIAquick column and centrifuged at 10000 rpm for 30–60 seconds. After discarding the flow-through, the QIAquick column was placed back into the same tube and washed with 0.75 ml of Buffer PE by centrifugation at 10000 rpm for 30–60 seconds. After discarding the flow-through, column was centrifuged for an additional 1 minute at maximum speed. Finally, QIAquick column was placed in a clean 1.5 ml microcentrifuge tube and 50 µl of sterile distilled water was used to elute DNA by centrifugation at maximum speed for 1 minute.

In order to purify linearized plasmid from agarose gel, a QIAquick Gel Extraction Kit was used. This kit is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. The DNA fragment contained in the agarose gel was excised with a clean, sharp scalpel then the gel slice was weighed in a colorless tube. After that, 3 volumes of Buffer QG was added to 1 volume of gel (100 mg ~ 100 µl) and incubated at 50 °C for 10 minutes (or until the gel slice has completely dissolved), the tube was mixed by vortexing every 2–3 minutes during the incubation to help dissolving the gel. After the gel slice has dissolved completely, it was necessary to make sure that the color of the mixture was yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture was orange or violet, added 10 µl of 3 M sodium acetate, pH 5.0 and mixed. The color of the mixture would turn to yellow. The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding. Next, 1 gel volume of isopropanol was added to the sample and mixed. Then the sample was applied to the QIAquick column, and centrifuged 10000 rpm for 1

minute. After the flow through was discarded, QIAquick column was placed back in the same collection tube. To remove all trace of agarose, 0.5 ml of Buffer QG was added to QIAquick column and centrifuge for 1 minute. To wash, 0.75 ml of Buffer PE was added to QIAquick column and centrifuge for 1 minute. Discard the flow-through and centrifuge the QIAquick column for an additional 1 minute at 13000 rpm. After placing the QIAquick column into a clean 1.5 ml microcentrifuge tube, 50 µl of steriled water was added to the center of the QIAquick membrane to elute DNA and centrifuged for 1 minute at maximum speed.

1.2.2 Ligation

The ligation of digested NdeI-p53-EcoRI DNA fragment into pAK400 vector was performed using T4 DNA ligase enzyme. The ligation mixtures consist of 5 ng of NdeI-p53-EcoRI DNA insert, 90 ng of pAK400 vector and 2 µl of 10X ligase buffer (200 mM Tris-HCl, 100 mM $MgCl_2$ and 250 µg/ml acetylated BSA) 5 units of T4 DNA ligase was added last and gently mixed by stirring with pipette tip. The ligation reaction was incubated at 16 °C overnight.

1.2.3 Preparation of competent *E. coli* cells for transformation

Streak-plate of *E. coli* cells [XL-1blue, BL21(DE3) or BL21(DE3)pLysS] were prepared using LP tes. The plates were then incubated at 37 °C overnight. After that, a single *E. coli* colony was inoculated in 10 ml LB-broth (in 25 ml volumetric flask) and cultured in a 37 °C shaker at 180 rpm until the bacteria reached $OD_{600\ nm}$ = 0.8. Next, cultured bacteria was transferred to a 15 ml centrifuge tube and centrifuged at 4 °C for 10 minutes at 2500 rpm (10000 g). Then bacterial pellet was resuspended in 10 ml ice-cold 0.1 M $CaCl_2$ and centrifuged again as above. Afterthat

pellet was resuspended in 10 ml ice-cold 0.1 M $CaCl_2$ again, and stood on ice for 1 hour. Competent cells can be obtained with incubation times on ice as low as 20 minutes, but the longer times greatly increase the overall transformation efficiency. Before cells were recentrifuged as above. Pellet was resuspended in 4 ml ice-cold 0.1 M $CaCl_2$ and mixed with 800 µl sterile glycerol. Before being aliquoted 200 µl each into 1.5 ml eppendorf tubes and stored at -70 °C until used. Bacteria prepared in this manner can be successfully used after up to one year's storage and will still maintain high transformation efficiency.

1.2.4 Verification of ligation

Before attempting transformation of *E. coli* with the pET-15b-p53 ligation products, it was necessary to verify whether the appropriate ligation had occurred. An amount of ligation product was removed and subjected to PCR amplification using the same pair of primers used to generate PCR product NdeI-p53-EcoRI, the pair of T7 promoter primer (T7 promoter locates in pET-15b vector) and p53BamHI primer (primer which use to amplify p53 gene), the pair of NdeIp53 primer and T7 terminator and the pair of T7 promoter and T7 terminator. The PCR mixture contents were the same as described in Table 1.

1.2.5 Transformation of competent cells

After ensuring that appropriate ligation had occurred, the ligation mixture obtained from section 1.2.2 was then used for transformation into *E. coli* strain XL-1blue cells. Firstly, competent cell tubes was removed from the -80 °C freezers and immediately place the tube on ice, so that all but the cap was surrounded by ice. The cells were allowed to thaw on ice for ~ 2-5 minutes. Then 20 µl of ligation mixture

was added into 200 µl competent *E. coli* strain XL-1blue and stirred gently to mix and returned the tube to the ice. After cells were incubated on ice for 1 hour, the mixture was transferred into a cold screw cap glass tube. Following transferring, the cells were heat shocked by removing tubes from the ice and immediately immersing them in 42 °C water bath for 90 seconds, after which they were placed back into the ice, immediately. After the tube had sat on ice again for 1 minute, 3 ml SB broth (see appendix) was added to the tube and cells were allowed to grow at 37 °C for 3 hours with 130 rpm shaking rate. After 3 hours, cells were centrifuged at 2500 rpm (22 °C) for 10 minutes to discard old medium and the pellet was resuspended in 500 µl SB broth. Then 50, 150 and 300 µl of bacterial suspension was spreaded onto each LB plate containing 50 µg/ml chloramphimicol (see appendix) and bacterias were allowed to grow overnight at 37 °C.

1.2.6 Positive clones screening

Only bacteria which uptake pAK400 plasmid vector would carry a chloramphenicol resistance gene making them resistant and could grow on agar medium containing chloramphenicol. However the resistant colonies were needed to be screened whether they have received pAK400 vector with right inserted DNA. Bacterial colonies were subjected to screening with PCR using NdeIp53 primer and p53EcoRI primer the same pair of primers used to generate PCR product NdeI-p53-EcoRI. The PCR mixture contents were the same as described in Table 1. The pAK400-p53 plasmid from transformant proved to carry the correct plasmid by PCR was purified from 10 ml overnight culture of recombinant *E. coli* XL-1blue cell using QIAGEN plasmid mini prep kit. In this procedure, bacterial cells are lysed under alkaline conditions and the crude lysates are cleared by centrifugation. The cleared

lysate is then loaded onto the anion-exchange tip where plasmid DNA selectively binds under appropriate low-salt and pH conditions. RNA, proteins, metabolites, and other low-molecular-weight impurities were removed by a medium-salt wash, and ultrapure plasmid DNA was eluted in high-salt buffer. The plasmid DNA was concentrated and desalted by isopropanol precipitation and collected by centrifugation. The obtained plasmid was then sequenced to check the orientation and in framed of the insert. The sequencing experiment was performed by the BioService Unit (BSU) of the National Center for Genetic Engineering and Biotechnology (Biotec) of Thailand. After confirmation, 5 ng of plasmid was transformed into the competent *E. coli* Origami B cells. The transformed *E. coli* Origami B which was used as the expression host for this DNA construct.

1.2.7 Growth of the correct transformant in liquid media and storage

The corrected clone was inoculated in 5 ml LB-broth cultures (in 10 ml screw-cap tubes) and shaked overnight at 37 °C in a shaker at 180 rpm. The culture then was mixed with 1 ml sterile glycerol. Cells were dispensed into 1 ml aliquots in 1.5 ml eppendorf tubes and stored frozen at -70 °C until used.

1.3 Cloning of p53 encoding DNA into pET-15b vector

1.3.1 NdeI-p53-BamHI and pET-15b restriction enzymes digestion

First, the pET-15b vector was double digested with *Nde*I and *BamH*I in order to linearize the super coiled plasmid. Meanwhile, NdeI-p53-BamHI PCR product was also digested with *Nde*I and *BamH*I to produce stricky ends available for the insertion into vector. For pET-15b enzyme digestion, a 500 µl reaction tube containing the 20 µl mixture consists of 2 µg of pET-15b vector, 20 units of *Nde*I, 20 units of *BamH*I

and 4 µl of 10X Tango buffer. For NdeI-p53-BamHI enzyme digestion, a 500 µl reaction tube containing the 10 µl mixture consists of 100 ng NdeI-p53-EcoRI PCR product, 5 units of *Nde*I, 5 units of *BamH*I, 2 µl of 10X Tango buffer. Both mixtures were incubated in 37 °C the water bath overnight. Prior to ligation, DNA insert and the linearized plasmid DNA was purified using QIAquick PCR purification kit. (As previously described in section 1.2.1)

1.3.2 Ligation

The ligation of digested NdeI-p53-BamHI PCR product into pET-15b was performed using ligase. The ligation mixture consists of 37 ng of NdeI-p53-BamHI DNA insert, 590 ng of pET-15b vector, 2 µl of ligase buffer and 5 units of T4 DNA ligase. Then the mixture was incubated overnight at 16 °C

1.3.3 Transformation

The transformation was carried out as previously described in section 1.2.5

1.3.4 Positive clones screening

The transformants were screened with PCR using T7 promoter primer and p53BamHI. The PCR mixture contents were the same as Table 1. After that, the pET-15b-p53 plasmid from the correct transformant was purified from 10 ml overnight culture of recombinant *E. coli* XL-1blue cell using QIAGEN plasmid mini prep kit (QIAGEN) and plasmid was confirmed by DNA sequencing. After confirmation, the correct plasmid were transformed into the competent *E. coli* BL21(DE3) cells and BL21(DE3)pLysS cells, which were later used as expression host.

2. Production of recombinant p53 protein

2.1 Optimization of culture conditions for the production of biotinylated p53-BCCP fusion proteins from pAK400 DNA construct.

In order to obtain the optimal temperature and post induction time, 10 µl of frozen cell stock of *E. coli* Origami B harboring the pAK400-p53 plasmid was inoculated into 10 ml of SB media (see appendix) containing 50 µg/ml chloramphenicol at 37 °C with shaking at rate approximately 180 rpm until OD_{600nm} reached 0.6-0.8. One ml of the culture was harvested aseptically and pelleted by centrifugation at 3000 rpm for 10 minutes. The pellet was stored at −20 °C until analyzed. This fraction was considered as the non induced sample and will be referred as, T0. At the same time, an aliquot of 2 ml of the bacterial culture was added into three of 125 ml flask containing 25 ml of SB medium (containing 50 µg/ml chloramphenicol) and allowed to grow at 37 °C until OD_{600nm} reached 0.6-0.8. Afterward, each the culture flask was activated with 25 µl of 1 M of isopropyl-β-d-thiogalactopyranoside (IPTG) and 10 µl of 4 µM biotin. The culture flask was separately cultured at different temperature with shaking (180 rpm) at 25, 30 and 37 °C, respectively. During the first 7 hours, 1 ml of the induced culture was harvested every 1 hour and finally at 21 hours. These fractions were considered as the T1-T7 and T21 sample, respectively. Pellets were stored at −20 °C until used. Non-transform *E. coli* Origami B were used as the negative control (Neg). For the preparation of *E. coli* lysate, the bacterial cell pellets (T0, T1-T7, T21, and Neg) were resuspended in 150 µl of B-PERII lysis buffer (PIERCE) containing protease inhibitor. After that, the cell pellet was vortexed on ice for 1 minute. The cell lysate

was then centrifuged at 10000 *g* for 10 minutes at 4 °C and the supernatant was collected into fresh microcentrifuge tubes and stored at −20 °C until analysis.

2.2 Optimization of culture conditions for the production of $(His)_6$-p53 fusion protein from pET-15b DNA construct.

Five microliltres of frozen cell stock of *E. coli* BL21(DE3) and BL21 (DE3) pLysS harboring the pET-15b-p53 plasmid was inoculated the same way as previously described in section 2.1, excepted ampicillin (200 μg/ml) was added into SB media instead of chloramphenicol and biotin was left out.

2.3 Determination of the level of expressed protein

Protein samples from the supernatant and cell lysate was analyzed by Western blot analysis. The details of procedure are as followed

2.3.1 Protein assay

The BCA™ Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000 μg/ml). Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). Series of dilutions of known

concentration are prepared from the protein and assayed alongside the unknown(s) before the concentration of each unknown is determined based on the standard curve.

Pipette 10 µl of each standard or unknown sample triplicate into a microplate well. After that, 190 µl of the reagent mixture containing reagent A and B in ratio 50:1 were added to each well and mixed thoroughly on a plate shaker for 30 seconds. Reaction plate was incubated at room temperature for 30 minutes. The absorbance at or near 550 nm was measured using a microplate reader. A standard curve between the average Blank-corrected 550 nm measurements for each BSA standard vs. its concentration in µg/ml was generated and used to determine the protein concentration of each unknown sample.

2.3.2 Separation of protein by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS-PAGE, officially *s*odium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique used in biochemistry and molecular biology to separate proteins according to their size (length of polypeptide chain or molecular weight). The addition of SDS, proteins may optionally be boiled in the presence of a reducing agent, such as dithiothreitol (DTT) or 2-mercaptoethanol (beta-mercaptoethanol / β-ME), which further denatures the proteins by reducing their disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (oligomeric subunits).

In this study, a 15% polyacrylamide separating gel (prepared by combining 7.5 ml of 30% acrylamide solution, 3.75 ml of 1.5M Tris-HCl buffer pH 8.8, 75 µl of 10% SDS, 5 µl of 10% ammonium persulfate, 5 µl of TEMED and 3.45 ml of deionized water) topping with 4% polyacrylamide stacking gel (prepared by combining 665 µl of 30% acrylamide solution, 1.25 ml of 0.5M Tris-HCl buffer pH

6.8, 50 µl of 10% SDS, 25 µl of 10% ammonium persulfate, 5 µl of TEMED and 3 ml of deionized water) was used to separate proteins from cell lysate. Separating gel ingredients were mixed and filled into gel sandwich until reaching the desired level, the gel was allowed to polymerize for 40 minutes at room temperature. After that stacking gel solution was mixed and filled on top of the separating gel. Then the comb was insert into stacking gel and allowed gel to polymerize for 30 minutes.

After obtaining the concentration of each cell lysate, loading mixture was prepared by combining 20 µg protein cell lysate with 2X loading buffer (containing 0.5 M Tris-HCl pH 6.8, 20% (v/v) glycerol, 2% (v/v) 2-ME and 0.005% (w/v) bromphenol blue) in ratio 1:1 and loaded to each well of the gel. Separation of protein in cell lysate on SDS-PAGE was performed at a constant voltage of 180 Voltages for 1.5 hours. Electrophoresis was terminated when the position of tracking dye which monitors the progress of the run reached the bottom of the gel.

2.3.3 Electrotransfer of separated proteins from the gel into the PVDF membrane

While the gel was running, PVDF (PALL, USA) membrane was prepared for blotting by soaking in methanol for 1 minute, in deionized water for 5 minutes and transferring buffer (see appendix) for at least 15 minutes. After that, two pieces of fiber pad, two filter papers were soaked in transfer buffer. After the electrophoresis was terminated, the gel was removed for transferring. The following items were assembled in order to start blotting, from the black side of the blotting cassette: fiber pad, filter paper, gel, PVDF membrane, filter paper, fiber pad and the red cassette clamp, respectively. The cassette was placed in the transfer tank, the black side was closed to the negative electrode and the transferring buffer (25 mM Tris-base,

200 mM glycine and 20% (v/v) methanol, and 0.037% SDS) was filled until reaching the top of cassette. Electroblotting was performed by applying 30 voltages overnight.

After transferring separated proteins from polyacrylamide gel onto PVDF membrane, the membrane was blocked with TBS-T (20 mM Tris-HCl pH 7.5, 100 mM NaCl and 0.05% tween-20 containing 5% skimmed milk (Mission) at room temperature for 1 hour. Then, the membrane was incubated with a 1:1000 dilution (v/v) of the monoclonal antibodyanti-p53 monoclonal antibody(DO7) (Novocastra, UK) in dilution buffer (5% skimmed milk in TBS-T) at room temperature for 1 hour. After the membrane was subjected to 6 rounds of 10 minute-wash with TBS-T, 1:1000 dilution (v/v) of horserashdish peroxidase conjugated goat anti-mouse immunoglobulin antibody (DakoCytomation) in dilution buffer was added. Finally, after extensive washing, the targeted protein band was detected by ECL Western blotting detection reagents.

3. Purification of $(His)_6$-p53 protein under denaturing conditions

3.1. Purification of $(His)_6$-p53 protein by His•Bind Resin chromatography

An overnight culture of a positive clone was harvested by centrifugation and the cell pellet was resuspended in lysis buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, 6 M urea, pH 7.9) at 10 ml lysis buffer per 40 ml cell culture. The cell suspension was vortexed for 1 minute at room temperature until homogeneously. The cell lysate was recentrifuged at 10000 *g* for 10 minutes and the supernatant was transferred to a fresh tube to further purified by using the His•Bind Resin (Novagen). Firstly, a bottle of His•Bind Resin was gently mixed by inversion until completely suspended. Afterthat a widemouth pipet was used to transfer 5 ml of 50% His•Bind Resin slurry to column and allow resin to pack under gravity flow. When level of

storage buffer drops to top of column bed, used the following sequence of washes to charge and equilibrate column:

a) 3 volumes of sterile deionized water.

b) 3 volumes of 1X Binding Buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, 6 M urea, pH 7.9).

After 1X Binding Buffer was drained to top of the column bed, 80 ml of prepared cell lysate was loaded to column, the first flow through designated as lane 2. A flow rate of approximately was adjusted to 10 volumes per hour as it was recommended to be optimal for efficient purification. If flow rate is too fast, more impurities will contaminate the eluted fraction. The column was washed with 10 volumes 1X Binding Buffer and 6 volumes 1X Wash Buffer (0.5 M NaCl, 80 mM imidazole, 20 mM Tris-HCl, 6 M urea, pH 7.9), washed fractions were designed as FT_1 and FT_2 respectively. The recombinant protein was eluted twice with 10 volumes 1X Elute Buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and these eluted fractions were marked as E_1 and E_2. Finally, 6 volumes 1X Strip Buffer (0.5 M NaCl, 100 mM EDTA, 20 mM Tris-HCl, pH 7.9) was used to wash Ni^{2+} from column and used resin was kept in stripping buffer, this fraction marked as S. The 15 µl of each collected fractions were mixed with 15 µl of 2X loading buffer and analyzed by SDS–PAGE (as described in section 2.3.2) for the determination of the eluted $(His)_6$ recombinant protein. After that, gel was subjected to Coomassie blue staining for the visualization of bands indicating the $(His)_6$-recombinant protein content in the gel. Coomassie (also known as Brilliant Blue, Brilliant Blue G, Acid Blue 90, C.I. 42655, or Brilliant Blue G 250) is a blue dye commonly used in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was soaked in dye for 30 minutes and then destained for 30 minutes or more with Destain I,

before the gel was destained with Destain II until the background of gel was clear. This treatment allows the visualization of bands indicating the protein content of the gel. The visualization on the gel usually contains a set of molecular weight marker so that protein MW can be determined in an unknown solution.

3.2. Dialysis of protein

Ten milliliters of the purified $(His)_6$-protein fraction was dialyzed using Dialysis tubing cellulose membrane (MW 10000) at 4 °C against 1 L PBS buffer pH 7.4 (10 mM phosphate buffer, 150 mM NaCl and 0.1% sodium azide, pH 7.4). Changed twice the first 6 hours and continued overnight. After that, the purified $(His)_6$-p53 was dispensed into 1 ml aliquots in 1.5 ml eppendorf tubes and stored frozen at -20 °C until used.

4. Optimization of ELISA

In principle, ELISA is a sensitive assay to quantitate picogram to microgram quantities of substances (such as antibodies, hormones, cell signaling chemicals, infectious disease antigens and cytokines.). In this study the p53 recombinant protein were used to set up an ELISA to detect p53 autoantibodies in cancer patients.

4.1. Optimization of ELISA conditions using biotinylated p53 BCCP fusion protein as an antigen

In principle, biotinylated p53 recombinant protein was expressed and biotinylated *in vivo* in *E. coli* Origami B strain and a crude cell lysate was applied directly to an avidin coated plate to allow a single step purification of the antigen through its biotinylated moiety. Afterward, unknown sera expected to have p53 autoantibodies or the commercial anti-p53 monoclonal antibody was applied onto

coated plate to allow p53 antibodies to bind with specific epitopes to the p53 recombinant protein. Then, an enzyme (HRP) labeled goat-antibody, specific to either mouse or human immunoglobulin was added to the p53-antibody complex. After washing off excess unreacted enzyme conjugate from the microwells, the substrate (TMB) was added and HRP labeled anti-immunoglobulin antibody would catalyse tetramethylbenzidin oxidation resulting in a colorimetric reaction. The color generated was measured spectrophotometrically at 450 nm. The intensity of the color developed was proportionally to the concentration of p53 autoantibodies.

4.1.1 Estimation of the optimal concentration of avidin and biotinylated p53 to be coated on ELISA plate

A 96-well plate was divided into 4 quadrants (as indicates in Figure 5) and each quadrant would use different concentrations of biotinylated p53 protein to identify the amount of p53 monoclonal antibody at one particular avidin concentration. As indicates in Figure 5, each quadrant of 96-well plate (Costa, corning Incorporated, USA) were coated with 100 µl of 5.0, 7.5, 10 and 15 µg/ml avidin dissolved in coating buffer (50 mM carbonate, pH 9.6), respectively, and incubated overnight at 4 °C.

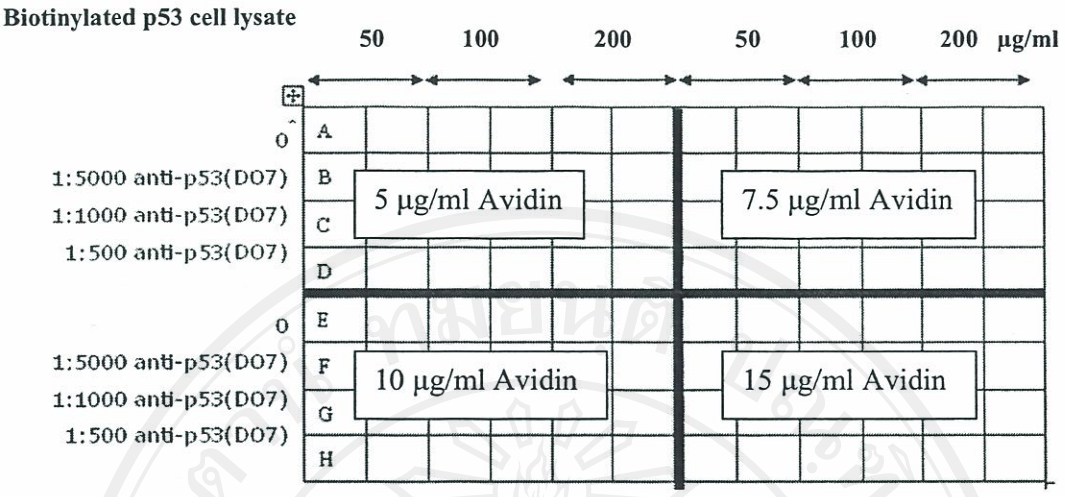

Figure 5. A 96-well plate which was divided into 4 quadrants and each quadants would used different concentrations of avidin and biotinylated p53 protein to identify the amount of p53 monoclonal antibody.

After discarding the coating solution and washed with PBS-T (10 mM phosphate buffer, 150 mM NaCl, 0.05% Tween 20) for four times, all of wells were blocked with 300 µl blocking solution (PBS-T containing 3% bovine serum albumin) and followed by four washings with PBS-T. Next, plates were incubated for 1 hour with 100 µl of 50, 100 and 200 µg/ml of the whole cell lysate of pAK400-p53 transform *E. coli* diluted in PBS-T buffer. After washing with PBS-T for four times, 100 µl of 0, 1:5000, 1:1000 and 1:500 of mouse anti-p53 (DO7) monoclonal antibody were added in row A-H as shown in Figure 6 and incubated for 1 hour before washing with PBS-T for four times. Afterward, 100 µl of 1:3000 diluted goat anti-mouse immunoglobulin-HRP conjugate (DAKO) was added and incubated for 1 hour. After extensive washing, 50 µl of substrate 3, 3, 5, 5, tetramethylbenzidine (TMB) substrate solution was applied into each well and incubated in the dark for 10 minutes at room temperature, before the reaction was stopped by the addition of 1N HCl. Optical density at 450 nm of each well was determined using microplate reader (Bio-TEK Instrument, USA) After obtaining the optimal concentrations of avidin to be coated

onto microplate, the ELISA was also performed with sera proven to be positive or negative for p53 autoantibodies by Western blot analysis at different concentrations of cell lysate as shown in Figure 6.

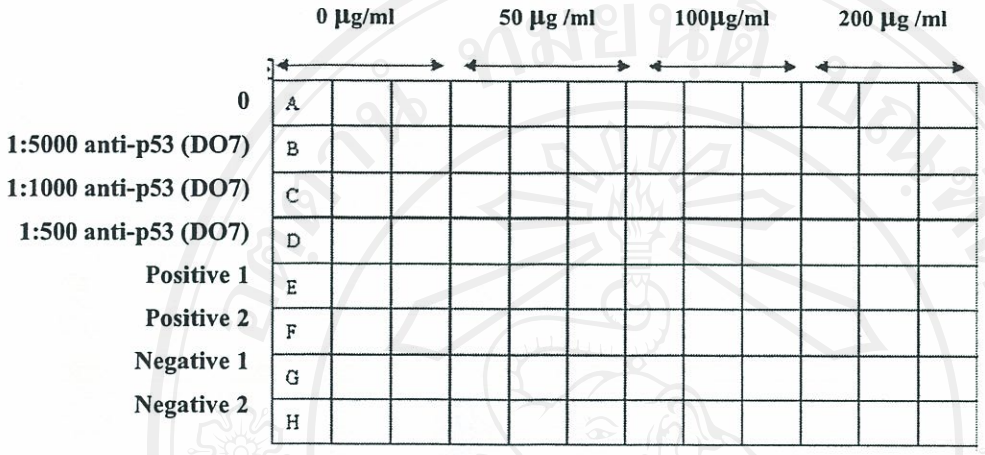

Figure 6. The ELISA was also performed with sera proven to be positive or negative for p53 autoantibodies by Western blot analysis at with the optimal concentration of avidin and different concentrations of cell lysate

4.2 Optimization of capture ELISA for $(His)_6$-p53

In principle, a purified p53 antigen is immobilized onto microwells. p53 specific immunoglobulin antibodies are allowed to react with the antigen. The excess unbound proteins are washed-off from the microwells. An enzyme (HRP) labeled goat-antibody; specific to immunoglobulin is added to the p53-antibody complex. After washing off excess unreacted enzyme conjugate from the microwells, a substrate (TMB) is added and HRP labeled anti-human immunoglobulin antibody catalyses tetramethylbenzidin oxidation resulting in a colorimetric reaction. The color generated is measured spectrophotometrically at 450 nm. The intensity of the color developed gives directly the concentration of p53 antibodies.

4.2.1 Optimization of His-tag-p53 concentration

ELISA plates were coated with 100 µl of 0, 1, 3 and 5 µg/ml of purified $(His)_6$-p53 protein in coating buffer and incubated overnight at 4 °C. After discarding the coating solution, plate was blocked with 300 µl of 5% skimmed milk dissolved in PBS-T for 1 hour. Then, different concentrations of the commercial p53 monoclonal antibody(0, 1:5000, 1:1000 and 1:500) mouse along with test sera (2 positive and 2 negative by western blot) diluted in PBS-T containing 5% skimmed milk to 1:200 were added in triplicates at 100 µl/well, and the plate was incubated for 1 hour. After washing with PBS-T for four times, 1:3000 diluted goat anti-mouse immunoglobulin-HRP conjugate or 1:5000 diluted rabbit anti-human immunoglobulin-HRP conjugate in dilution buffer was added and incubated for 1 hour. After extensive washing with PBS-T, 50 µl of TMB substrate solution (Zymed) was applied and the color development was stopped after 10 minutes by adding 50 µl of 1 N HCl before measuring absorbance 450 nm values using microplate reader.

4.2.2. Optimization of coating buffer for $(His)_6$-p53

In order to obtain the most suitable coating buffer for coating $(His)_6$-p53, ELISA was performed with 4 commonly used coating buffers. ELISA plates were coated with 5 µg/ml purified p53 diluted in different coating buffer, which included PBS pH 5.2 (50 mM PBS, pH 5.2), PBS pH 7.2 (50 mM PBS, pH 7.2), Tris pH 8.5 (50 mM TBS, pH 8.5) and carbonate buffer pH 9.6 (50 mM carbonate, pH 9.6) and incubated overnight at 4 °C. Afterward, coated plate was used to perform ELISA with different concentrations of anti-p53 monoclonal antibody and test sera as described in section 4.2.1.

4.2.3. Reproducibility testing of the established ELISA

Optimal condition variation (OCV) or within run assay variation is a statistic value calculated from the results of 20 replicates in a single run. In this study, the optimal condition variation (OCV) of the established method for p53 autoantibody detection was calculated from 20 assay wells of ELISA performing with control serum in a single run.

Routine condition variation (RCV) or between run assay is a statistic value calculated form various in runs (vary in times). In this study, the RCV was determined by assaying control serum used to obtain OCV simultaneously with the experimental samples in each run. Precision test was evaluated from percentage of coefficient of variation (%CV) by using the following formula:

$$\%CV = \frac{SD \times 100}{Mean}$$