

CHAPTER III

I. RESULTS

1. Construction of expression vectors (pET-15b-p53 and pAK400-p53)

In order to amplify the full-length p53 gene from pcDNA 3.1, PCRs were performed using a set of primer including NdeIp53 primer carrying restriction sequence for *NdeI* at 5' terminal in combination with p53EcoRI primer or p53BamHI primer possessing restriction sequence for *EcoRI* or *BamHI* at 3' terminal, respectively. From this experiment, the NdeI-p53-EcoRI DNA fragment which carrying the *NdeI* and *EcoRI* restricted site and NdeI-p53-BamHI which carrying the restriction site for *NdeI* and *BamHI* at the terminus of p53 encoding gene were generated. To confirm the PCR amplification, the PCR product was fractionated using 1.5% agarose gel electrophoresis. Successful amplification of the NdeI-p53-EcoRI and NdeI-p53-BamHI were clearly indicated by the presence of a strong band of approximately 1500 bp (Figure 7). After that, NdeI-p53-EcoRI and NdeI-p53-BamHI PCR products were purified using PCR purification kit and followed by double digested with *NdeI* and *EcoRI* for NdeI-p53-EcoRI DNA fragment or *NdeI* and *BamHI* for NdeI-p53-BamHI DNA fragment to produce sticky ends. Meanwhile, pAK400 vector and pET-15b vector were double digested with *NdeI* and *EcoRI* or with *NdeI* and *BamHI*, respectively to linearize the supercoiled plasmid. Successful plasmid digestion of the pAK400 vector and pET-15b vector were verified as indicated in Figure 8 and Figure 9, respectively. After obtaining the purified DNA fragments and vectors, the digested NdeI-p53-EcoRI DNA fragment

was then inserted and ligated into the *NdeI* and *EcoRI* treated pAK400 vector and the digested *NdeI*-p53-*BamHI* DNA fragment was inserted and ligated into the *NdeI* and *BamHI* treated pET-15b. In order to confirm that the appropriate ligation had occurred, the ligation products were verified using various pairs of primer (as described in CHAPTER II section 1.2.4). Successful ligations were confirmed by the presence of PCR products in lane 3-6 of Figure 10. In lane 3, the PCR product was generated from ligation mixture using *NdeI*p53 and p53*BamHI* primers (the pair of primer which used to generate p53 gene), which is consistent with the expected size of the inserted p53 DNA around 1.5 kb. Lane 4 and 5 show PCR products which were generated by the pair of T7 promoter primer (a forward primer specific for pET-15b vector) and p53*BamHI* primer and the pair of *NdeI*p53 primer and T7 terminator primer (a reverse primer specific for pET-15b vector). The higher molecular weight bands presented in lane 4 and 5 indicated that p53 encoding DNA was inserted into pET-15b vector. Lane 6 shows PCR products which were generated using the pair of T7 promoter and T7 terminator primer. Two strong bands presented in this lane with 1500 and 250 bp in size are believe to be the amplified products from self ligated pET-15b vector and the vector with p53 gene inserted, respectively. Taken all together, the result in Figure 10 demonstrated that the desired ligation product had occurred. After ensuring the appropriate ligation, the mixture was then used to transform *E. coli* strain XL-1blue cells. The chloramphenicol resistant colonies and ampicillin resistant colonies were selected in order to screen for the pAK400-p53 and pET-15b-p53 transformed clones, respectively. The resistant colonies were then subjected to PCR with the same pairs of primer used to generate the inserted DNA. Five out of eight clones screened were found to have up taken pAK400-p53 construct by the present of the expected size of PCR products (~1500 bp) and 2 out of 3 clones were found to have up-taken pET-15b-p53 construct (present of ~1500 bp PCR

products), as shown in Figure 11. In order to confirm the PCR results, plasmids were extracted from these positive clones and subject to digestion with the same pairs of restriction enzymes used to generate sticky end before the ligation process (*NdeI* and *EcoRI* for pAK400-p53 and *NdeI* and *BamHI* for pET-15b-p53). The same DNA constructs were also sent to BioService Unit (BSU, NSTDA) in order to perform DNA sequencing analysis. In Figure 12, the present of DNA band at molecular weight around 4000 bp of the remaining pAK400 vector and 1500 bp of the p53 inserted DNA after digestion confirmed that the transformant have up taken the pAK400-p53 DNA construct. In the case of pET-15b-p53 DNA construct, digestion with *NdeI* and *BamHI* failed to release the 1500 bp inserted p53 gene. However, the DNA sequencing result from BSU confirmed that both of the obtained clones carried the corrected pAK400-p53 and pET-15b-p53 DNA construct. Therefore, both of the DNA constructs were further used to produce recombinant proteins.

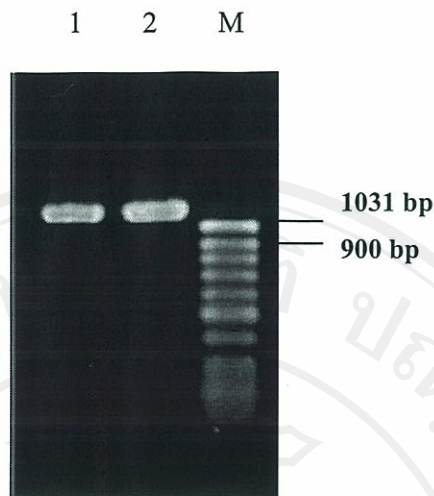


Figure 7. Agarose gel analysis showing the obtained p53 PCR products using NdeI-p53 and p53EcoRI as primers which was referred as “NdeI-p53-EcoRI” and from NdeI-p53 and p53BamHI primer which was referred as “NdeI-p53-BamHI” Lane 1 = NdeI-p53-EcoRI; lanes 2 = NdeI-p53-BamHI and Lane M = 1031-50 bp marker.

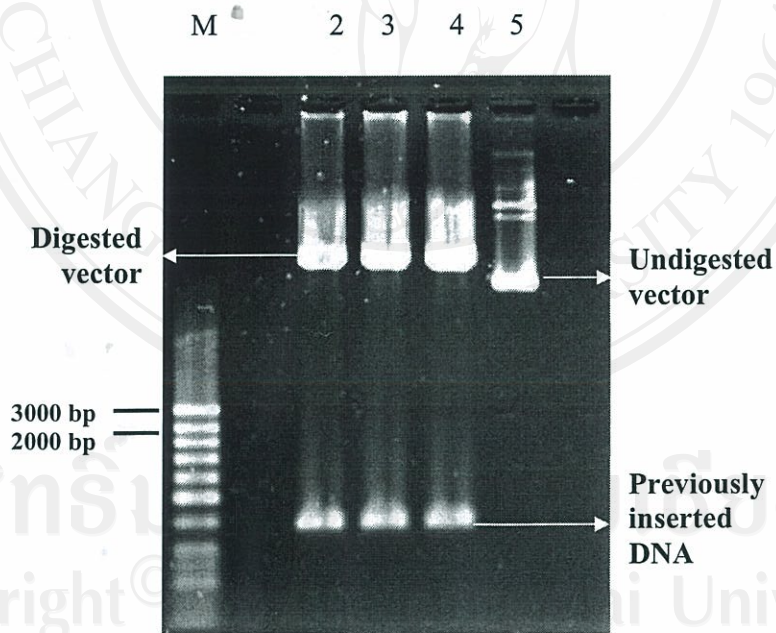


Figure 8. Agarose gel analysis showing successful digestion of previously inserted DNA harbouring pAK400 vector using *NdeI* and *EcoRI* restriction enzymes. Lane M = DNA marker, lanes 2-4 = *NdeI* and *EcoRI* treated pAK400 vector and lane 5 = undigested pAK400 vector.

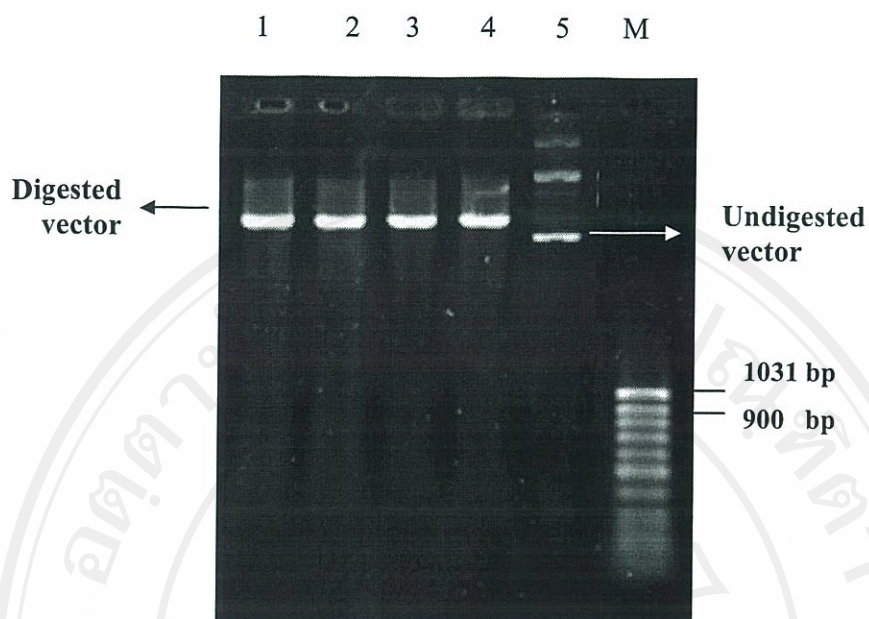


Figure 9. Agarose gel analysis of digested pET-15b empty vector using *NdeI* and *BamHI* on a 1.0% agarose gel. Lanes 1-4 = *NdeI* and *BamHI* treated pET-15b vector 100 bp marker, lane 5 = undigested pET-15b vector and Lanes M = DNA molecular weight marker.

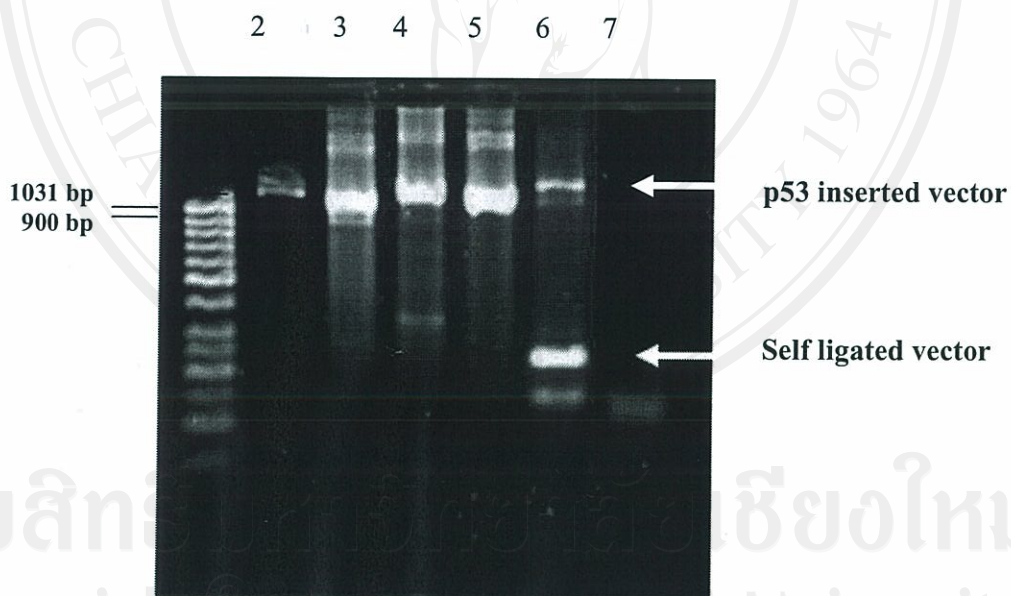


Figure 10. An example of agarose gel analysis of PCR products to verify the appropriate ligation between vector (pET-15b) and the inserted DNA.

- Lane M = DNA molecular weight marker
- Lane 2 = *NdeI*-p53-*BamHI* PCR product
- Lane 3 = amplified *NdeI*-p53-*BamHI* PCR product using *NdeI*p53 and p53*BamHI* primer
- Lane 4 = amplified *NdeI*-p53-*BamHI* PCR product using T7 promoter and p53*BamHI* primer
- Lane 5 = amplified *NdeI*-p53-*BamHI* PCR product using *NdeI*p53 primer and T7 terminator primer
- Lane 6 = amplified *NdeI*-p53-*BamHI* PCR product using T7 promoter and T7 terminator primer
- Lane 7 = negative control

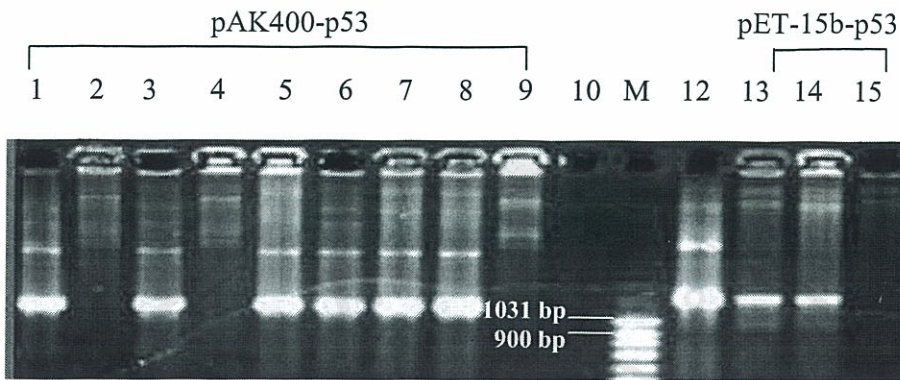


Figure 11. Agarose gel analysis of antibiotic resistant colonies screening by PCR.

- Lane 1-9 = the amplified PCR product from chloramphenicol resistant clones using *NdeI*p53 primer and p53*EcoRI* primer
- Lane 10 = negative control
- Lane M = 50 bp DNA marker
- Lane 12 = positive control for pET-15b-p53
- Lane 13-15 = the amplified PCR product from ampicillin resistant clones using T7 promoter primer and p53*BamHI* primer.

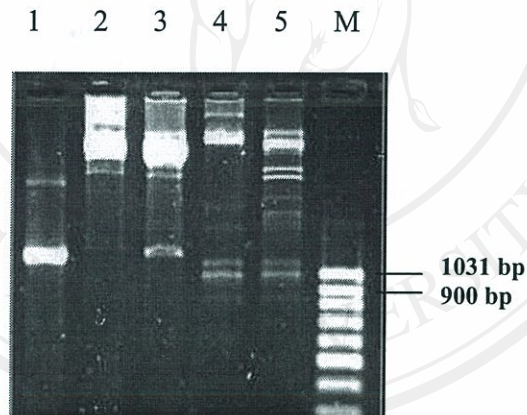


Figure 12 Agarose gel analysis of restriction enzyme digested products to confirm the obtained positive colonies.

- Lane 1 = *NdeI*-p53-*EcoRI* PCR product
- Lane 2 = the undigested plasmid which was extracted from a pAK400-p53 positive clone for PCR screening
- Lane 3 = *NdeI* and *EcoRI* digested pAK400-p53 plasmid
- Lane 4 = the undigested plasmid which was extracted from a pET-15b-p53 positive clone for PCR screening
- Lane 5 = *NdeI* and *BamHI* digested pAK400-p53 plasmid
- Lane M = DNA molecular weight marker

2. Production of p53 recombinant protein

2.1 Optimization of culture conditions for the production of biotinylated p53 – BCCP fusion protein from pAK400 DNA construct

The established pAK400-p53 DNA construct was transformed into an expression host *E. coli* strain Origami B. The expression levels of the biotinylated p53-BCCP fusion protein as a function of time was determined at various temperatures. The recombinant clone was cultured at 37 °C until OD₆₀₀ reached 0.8 when it was activated with 1 mM of IPTG. The expression cultivations were continued at various temperatures (25, 30 or 37 °C) during the induction phase. Parts of bacterial culture were taken at different post induction time points. Western blotting was performed to estimate expression level of the biotinylated p53 protein. The biotinylated p53 protein would have an estimated size of around 67 kDa (53 kDa of p53 protein plus 14 kDa of BCCP). As shown in Figure 13, the best quality of the biotinylated p53 was obtained at 25 °C. Although expression cultivation at 30 and 37 °C appeared to have higher protein quantity, too smaller p53 monoclonal antibody reactive protein products were obtained (Figure 13B and 13C). The optimal induction time was 6 hours after the IPTG induction. This is based on the high intensity band and clear from smaller p53 monoclonal antibody reactive protein fragment displayed in sample obtained at 6 hours post induction with IPTG (Figure 13A). Therefore, for the large scale cultivation (500 ml), this optimized condition was applied.

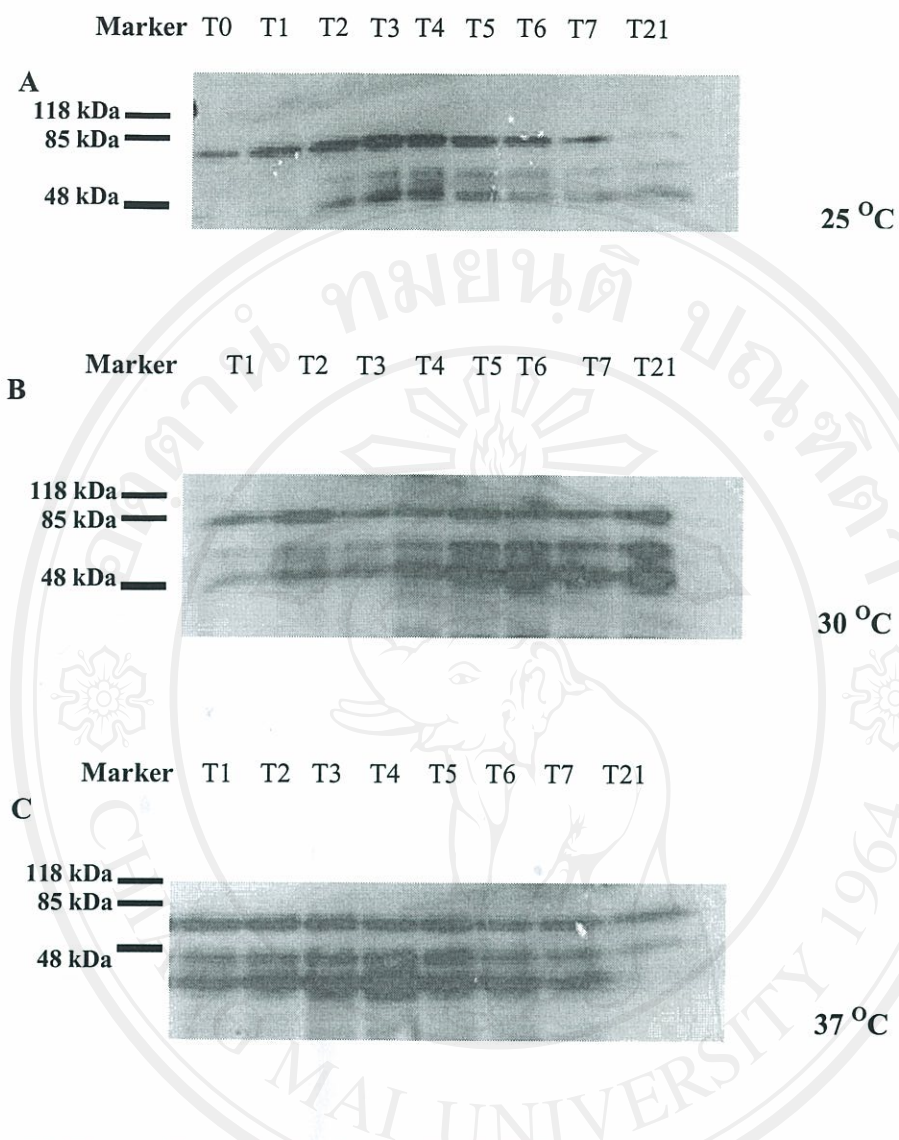


Figure 13. Western blot analysis of biotinylated p53-BCCP fusion protein expressed in *E. coli* Origami B cells. 20 μ g of cell lysates were electrophoresed and electroblotted onto PVDF membrane. The PVDF membrane was incubated with mouse anti-p53 monoclonal antibody (DO7). The expression cultivations were performed using the temperature of 25 °C (A), 30 °C (B) or 37 °C (C) during the induction phase 0-7 and 21 hours referred as T0-T7 and T21, respectively.

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2.2 Optimization of culture conditions for the production of (His)₆-p53 fusion protein from pET-15b DNA construct.

The recombinant pET-15b-p53 DNA construct was transformed into the expression host *E. coli* strain BL21(DE3) and BL21(DE3)pLysS which are specific bacterial strains designed for expression of genes regulated by the T7 promoter. The expression vector was chosen based on the presence of sequences encoding six histidine residues at 5' of the multiple cloning sites (MCS). The hexahistidine-p53 fusion protein would have an estimated size of about 53 kDa on SDS-PAGE. The expected distinct band around 53 kDa was observed in T0, T2, T4, T6, and T24 of the cell lysate samples. Furthermore, Western blotting analysis showed that basal expression of p53 could occur without IPTG induction since 53 kDa band can also be seen in the sample without IPTG induction (T0 in Figure 14, 15) especially in BL21(DE3) (T0 in Figure 14). However, IPTG induction can greatly increase the level of expression. The expected 53 kDa band was absent in the negative control (Neg in Figure 14, 15). Therefore, the Western blotting results confirmed that the p53 protein was successfully expressed in *E. coli* BL21(DE3) and BL21(DE3)pLysS. The optimal incubation time and temperature for the His₆-p53 protein expression was 24 hours after the IPTG induction at 25 °C in host strain BL21(DE3)pLysS (T24 in Figure 15A). This is based on highest intensity band and clear of p53 fragment band which was displayed by the supernatant sample. Therefore, for the large scale purification of expressed protein, this optimized condition was applied.

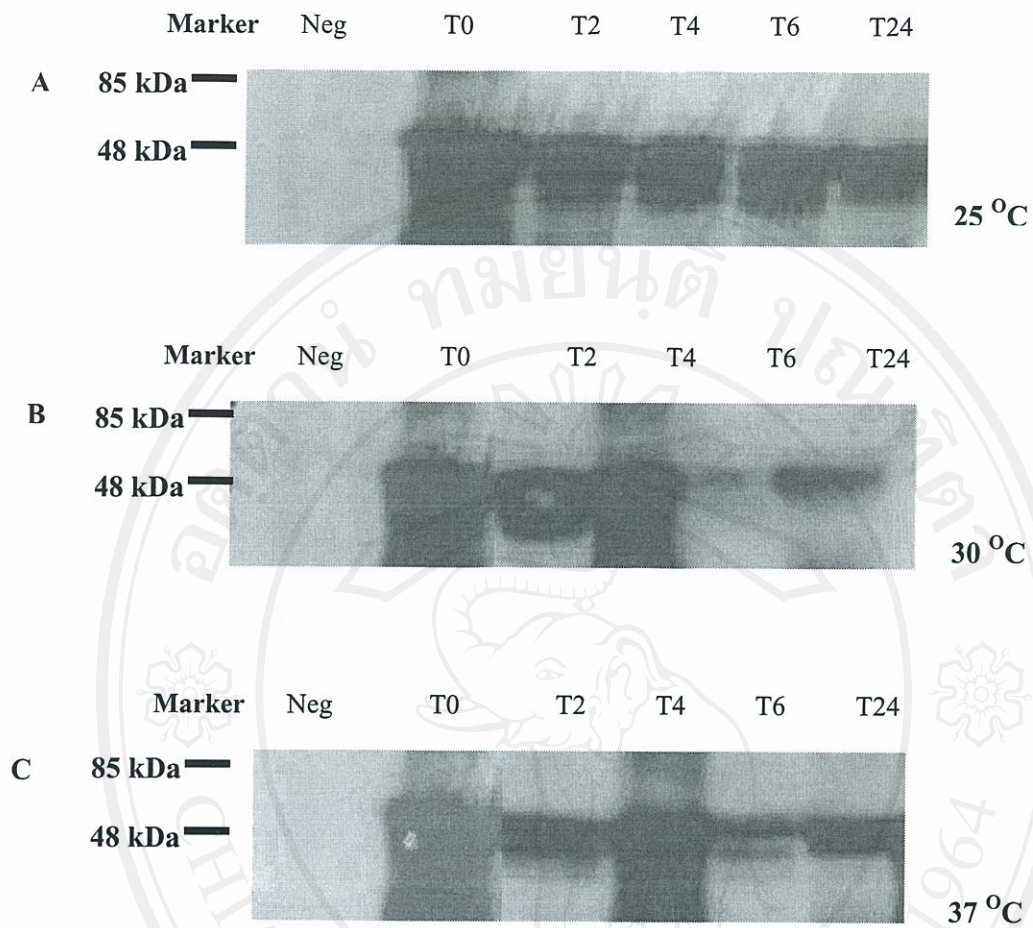


Figure 14. Western blot analysis of His₆-p53 fusion protein expressed in *E. coli* BL21(DE3). Twenty micrograms of cell lysates were electrophoresed and electroblotted onto PVDF membranes. The PVDF membrane was incubated with mouse anti p53 antibody (DO7). Non transformed bacterial cell lysate referred as Neg. The expression cultivations were performed using the temperature of 25 (A), 30 (B) or 37 (C) °C during the induction phase 0, 2, 4, 6 and 24 hours referred as T0, T2, T4, T6 and T24, respectively.

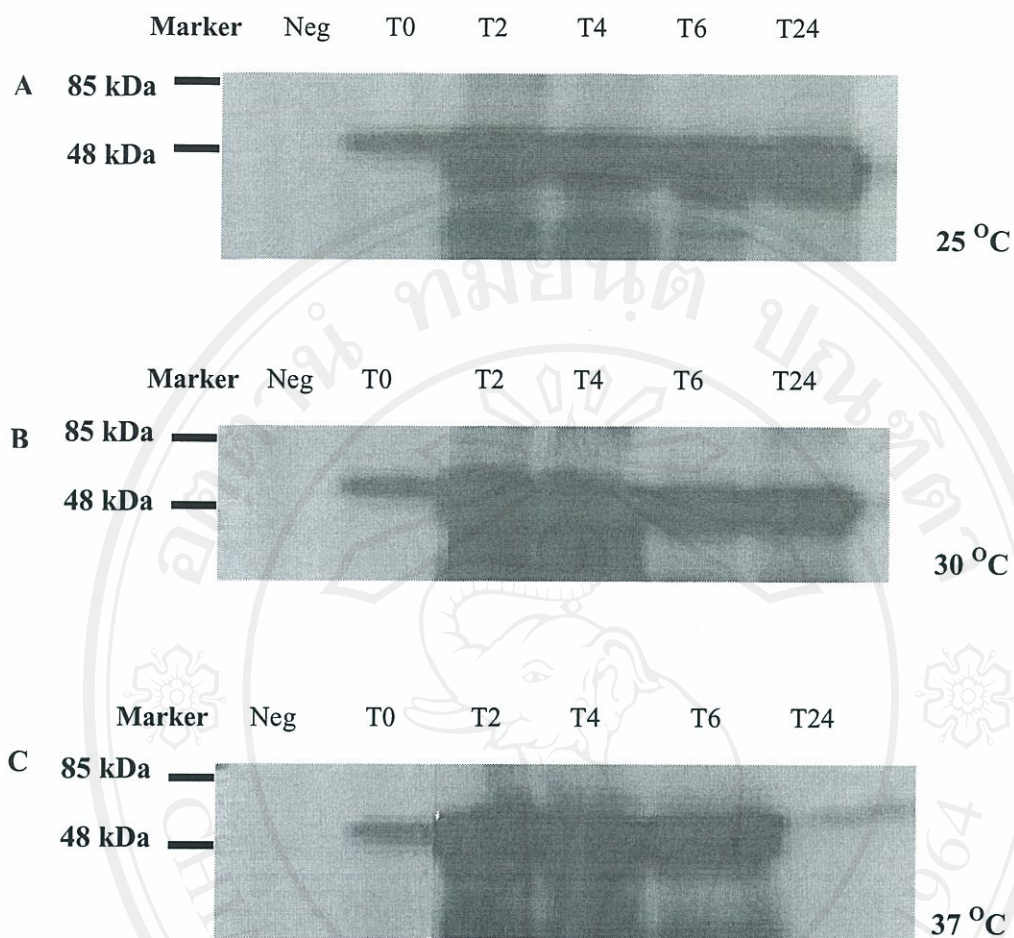


Figure 15. Western blot analysis of (His)₆-p53 fusion protein expressed in *E. coli* BL21(DE3)pLysS. Twenty micrograms of cell lysates were electrophoresed and electroblotted onto PVDF membranes. The PVDF membrane was incubated with mouse anti-p53 antibody (DO7). Non transformed bacterial cell lysate referred as Neg. The expression cultivations were performed using the temperature of 25 (A), 30 (B) or 37 (C) °C during the induction phase 0, 2, 4, 6 and 24 hours referred as T0, T2, T4, T6 and T24, respectively.

3. Purification of (His)₆-p53 protein under denaturing conditions

It is well recognized that an amino acid sequence consisting of 6 or more histidine residues in a row can act as metal binding site. Therefore, when target proteins were produced in fusion with His-tag sequence, they can be purified using a solid support that is covalently modified to display a heavy metal like Ni²⁺ on the surface. After producing (His)₆-p53 protein in a large-scale, bacterial cell lysate was prepared and purified with Ni²⁺-charged His•Bind resin under denaturing conditions using 6 M urea. Crude cell lysate was solubilized in 0.5X binding buffer and applied onto Ni²⁺-charged resin, after which it was washed with binding buffer containing 20 mM imidazole and washing buffer containing 40 mM imidazole before being eluted with 1 M imidazole. During this purification process, an aliquot of each step flow through was collected and subjected to SDS-PAGE and Coomassie blue staining. From Figure 16, the result shows that although wash buffer containing 40 mM of imidazole can get rid a lot of non-specific proteins bound to Ni²⁺-charged resin (lane 5, Figure 16), there are still a lot of proteins remained contaminant in the elution fraction (lane 6, Figure 16).

Therefore another Ni²⁺-chelated column chromatography was performed with washing buffer containing various concentration of imidazole (20 mM, 40 mM, 80 mM, 200 mM and 500 mM). As shown in Figure 17, the highest concentration of imidazole that can be used to wash out non-specific proteins bound to Ni²⁺-charged resin without eluting (His)₆-p53 is 80 mM. Thus for large scale purification 80 mM of imidazole was added into washing buffer.

During the purification step using optimal condition, fraction of flow-through was also collected and subjected to analysis, as shown in Figure 18. From Coomassie blue staining, the purified (His)₆-p53 was estimated to be around 0.25 µg/ml in comparison to

band intensity of the known amount of albumin protein standard loaded. In this study, from 500 ml of bacterial culture, 80 ml of cell lysate was prepared and subjected to affinity chromatography. For every 80 ml of cell lysate (from 160 ml of cell culture) subjected to purification through 5 ml of Ni²⁺-charged resin, around 1 mg of (His)₆-p53 was obtained. After the purification process, the obtained (His)₆-p53 was dialyzed against PBS, pH 7.4 at 4 °C overnight to get rid of imidazole.

The purified (His)₆-protein was further characterized by probing with mouse anti-human p53 monoclonal antibody (clone DO7) and the result is shown in Figure 19. The reactivity of the purified protein to p53 monoclonal antibody confirmed that the purified protein is in fact p53.

In order to set up an ELISA, it is necessary to identify sera which are positive for producing p53 autoantibody. Therefore, the purified (His)₆-p53 was resolved through SDS-PAGE, transferred onto PVDF membrane which was subsequently cut into small strip and probed with serum (diluted to 1:200) from lung cancer patients along with anti-p53 monoclonal antibody as a positive control.

As shown in Figure 20, sera from cancer patients which have reactivity band the same size as the positive control (p53 monoclonal antibody) is believed to have developed autoantibody against p53. From this, 1 weakly positive, 1 strongly positive, 1 clearly negative and 1 negative but gave a non-specific band were chosen and utilized to set up ELISA.

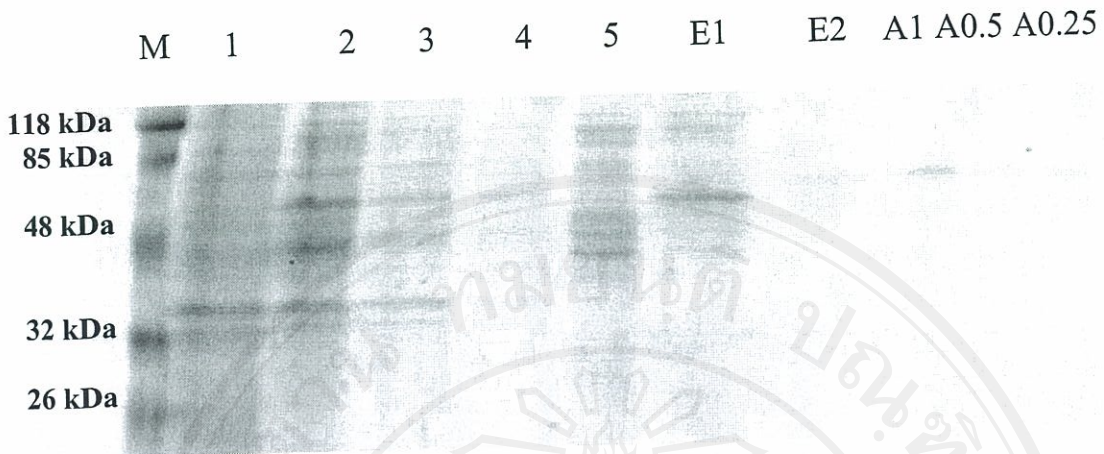


Figure 16 Coomassie blue stain of polyacrylamide gel showing preliminary purification process of His-p53 fusion protein using affinity chromatography. Fractions of flow-through were analyzed by SDS-PAGE, followed by Coomassie blue staining. The expected molecular mass of the purified protein was ~53 kDa.

- M = protein molecular weight marker
- Lane 1 = cell lysate from non transformed BL21(DE3)pLysS.
- Lane 2 = crude cell lysate from transformed bacterial total lysate containing (His)₆-p53 fusion proteins
- Lanes 3 = fraction of cell lysate which passed through the column
- Lane 4 = fraction of binding buffer containing 20 mM imidazole
- Lane 5 = fraction of washing buffer containing 40 mM imidazole
- Lane E1 = fraction of eluted His₆-p53 protein at 6 ml of elute buffer containing 1 M imidazole
- Lane E2 = fraction of eluted His₆-p53 protein at 3 ml of stripping buffer
- A1, A0.5, A0.25 referred as albumin at 1, 0.5, 0.25 µg/µl concentration.

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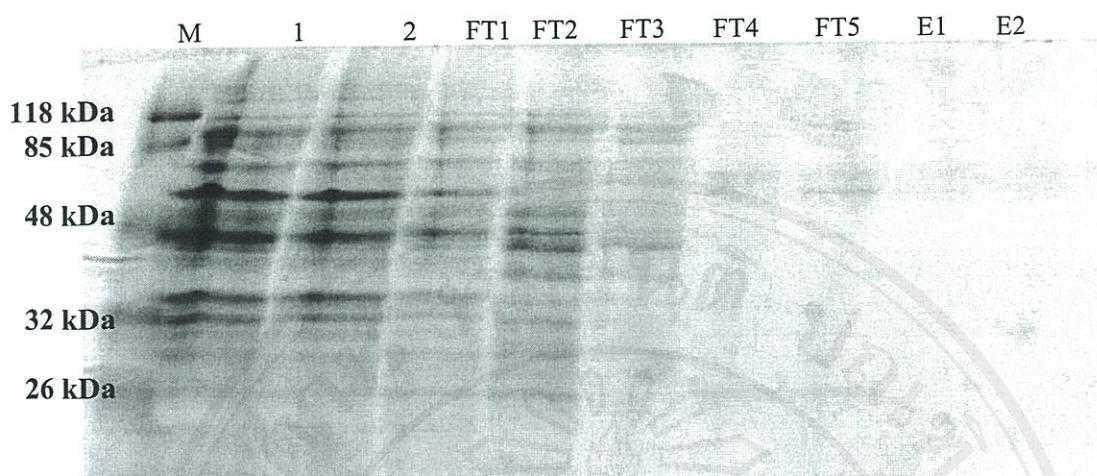


Figure 17 Coomassie blue stain of polyacrylamide gel showing effect of gradient concentrations of imidazole in washing buffer on the purification process. Fractions of flow-through were analyzed by SDS-PAGE, followed by Coomassie blue staining. The molecular mass of the purified protein was ~53 kDa.

- M Lane = molecular weight marker,
- Lane 1 = transformed bacterial total lysate containing (His)₆-p53 fusion proteins
- Lane 2 = fraction of cell lysate which flow through the column
- Lane FT1-FT5 = fraction of washing buffer containing 20, 40, 80, 200 and 500 mmol/l imidazole, respectively.
- Lane E1 = fraction of eluted His₆-p53 protein eluting with elute buffer containing 1 M imidazole
- Lane E2 = fraction of eluted His₆-p53 protein at eluted with stripping buffer

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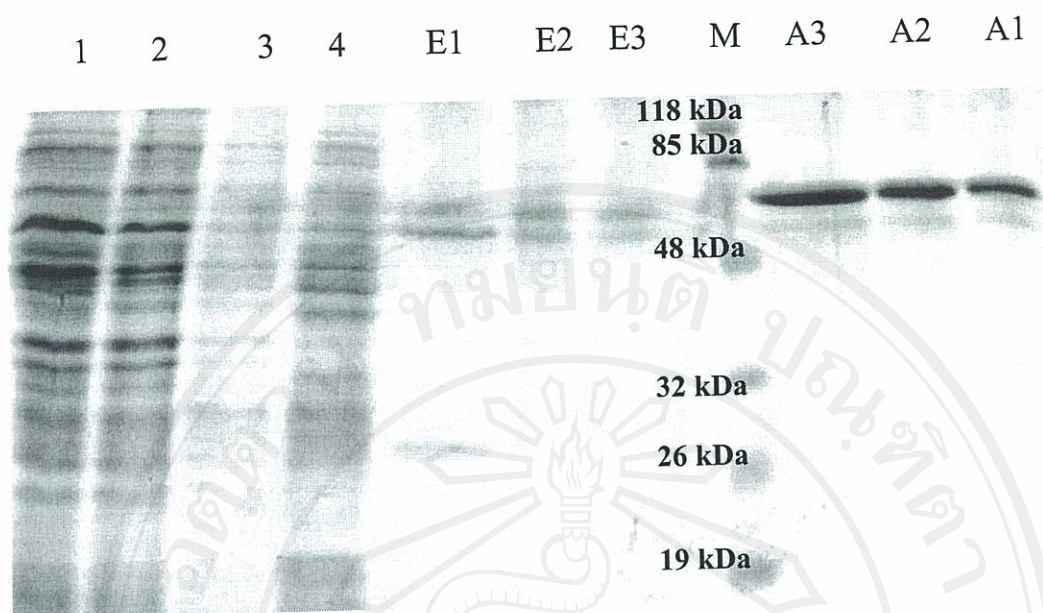


Figure 18 Coomassie blue stain of polyacrylamide gel showing purification process of His-p53 fusion protein using optimal condition. Fractions of flow-through were analyzed by SDS-PAGE, followed by Coomassie blue staining. The molecular mass of the purified protein was ~53 kDa.

- M = molecular weight marker
- Lane 1 = transformed bacterial total lysate containing (His)₆-p53 fusion proteins
- Lane 2 = fraction of cell lysate which flow through the column
- Lane 3 = fraction of binding buffer containing 20 mM imidazole
- Lane 4 = fraction of washing buffer containing 80 mM imidazole
- Lane E1 = fraction of eluted His₆-p53 protein at 10 ml of elute buffer containing 1 M imidazole
- Lane E2 = fraction of eluted His₆-p53 protein at 6 ml of elute buffer containing 1 M imidazole
- Lane E3 = fraction of stripping buffer
- A3, A2, A1 referred as albumin at 1, 0.5, 0.25 $\mu\text{g}/\mu\text{l}$ concentration.

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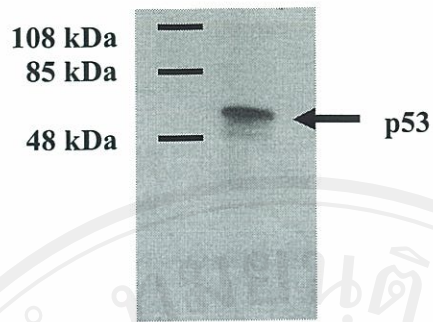


Figure 19. The purified (His)₆-protein was further characterized by probing with mouse anti-human p53 monoclonal antibody (clone DO7)

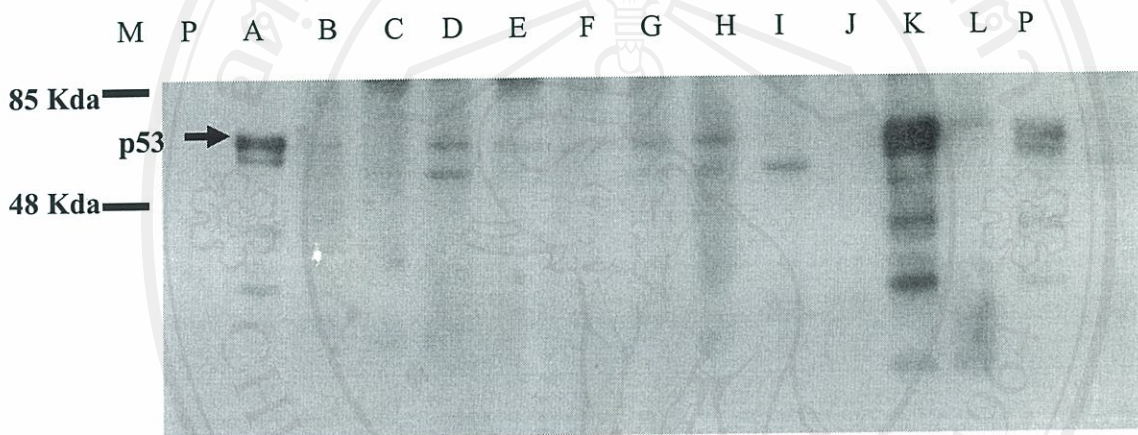


Figure 20. Representative results of mouse anti- p53 Abs of lung cancer patients detected by immunoblotting. PVDF membrane containing 3 µg per a strip of purified recombinant p53 was incubated with 1:5000 DO7 as positive control, 1:200 diluted sera from lung cancer, respectively.

- Lane M = molecular weight marker
- Lane P = positive control, probing with DO7
- Lanes A–L = probing with sera from lung cancer patients
 - Lane I = Negative 1
 - Lane H = Negative 2
 - Lane G = Positive 1
 - Lane J = Positive 2

4. Optimization of ELISA

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

In order to set up an ELISA for detecting p53 autoantibodies using biotinylated p53-BCCP, there are a few parameters that needed to be optimized, which included concentration of avidin and bacterial cell lysate to be coated onto microplate. For estimation of the optimal concentration of avidin to be coated, an ELISA plate was coated with various concentrations of avidin (5, 7.5, 10 and 15 $\mu\text{g/ml}$). This avidin coated plate was then subjected to pull out biotinylated p53-BCCP fusion protein from various concentration of crude bacterial cell lysate (0, 50, 100 and 200 $\mu\text{g/ml}$) and subsequently reacted with different dilution of p53 monoclonal antibody (0, 1:5000, 1:1000 and 1:500). The result showed that reactivity to p53 monoclonal antibody was increase when higher amount of bacterial cell lysate was applied onto microplate. However, different concentration of avidin (between 5-15 $\mu\text{g/ml}$) did not demonstrate any measurable effect on reactivity to p53 monoclonal antibody. Therefore future experiment was perform using the least amount as possible of avidin at 5 $\mu\text{g/ml}$ (Figure 21). ELISA plate coated with 5 $\mu\text{g/ml}$ of avidin and various concentrations of cell lysate was subject to react with serum (diluted to 1:200) from cancer patients (as chosen from Figure 20). From Figure 21, the result showed that human serum can react strongly to avidin coated plate. When various amount of biotinylated p53-BCCP contained bacterial cell lysate was coated, the reactivity was significantly increased. However, we found that biotinylated p53-BCCP failed to differentiate weakly positive serum from those that were negative.

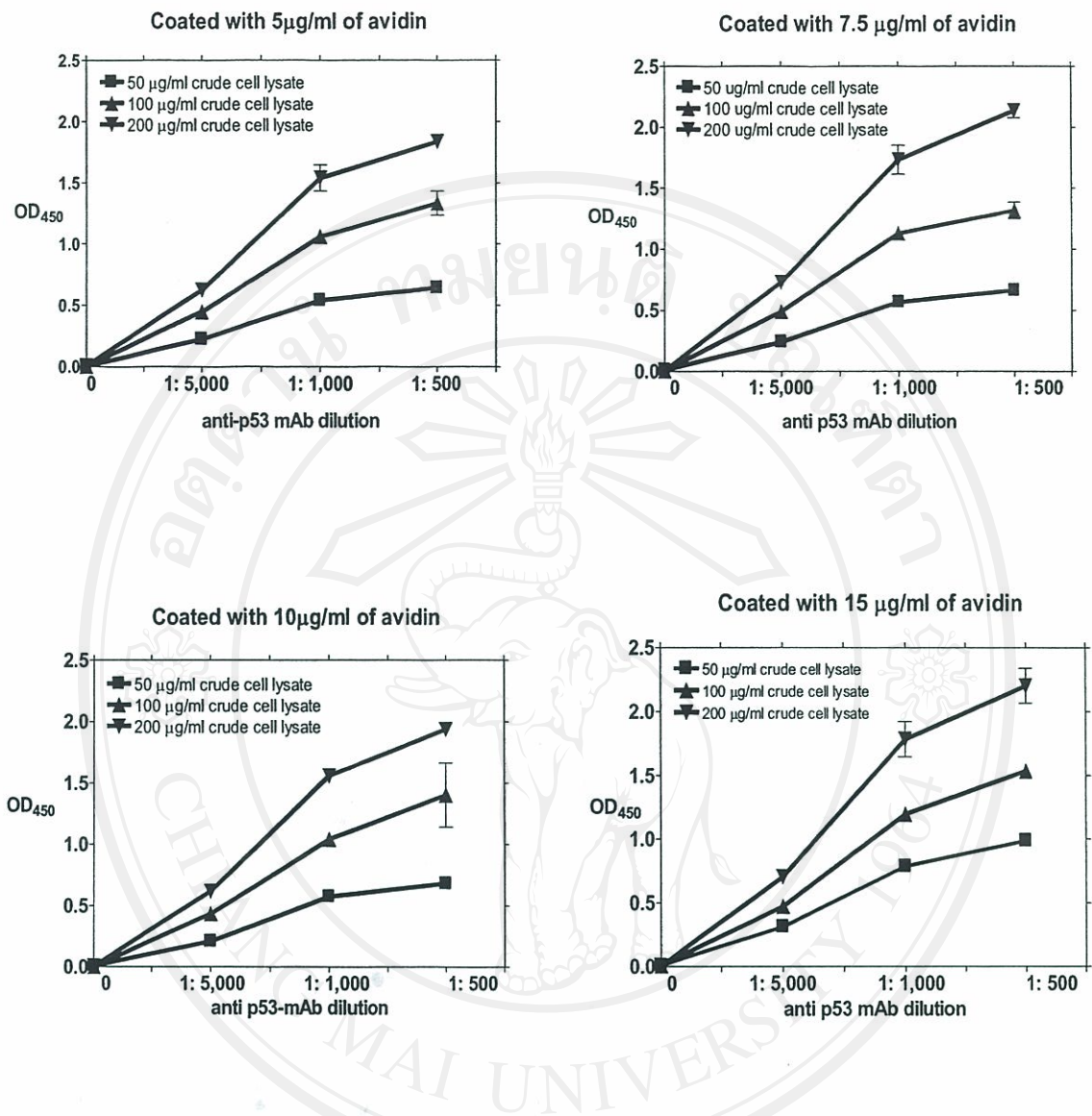


Figure 21. Optimization of the concentration of avidin and bacterial cell lysate containing biotinylated p53-BCCP protein to be coated onto microtiter plate. Various concentrations of avidin (5, 7.5, 10 and 15 µg/ml) and bacterial lysate (0, 50, 100 and 200 µg/ml) were tested for reactivity. Each point represents the mean value of two determinations.

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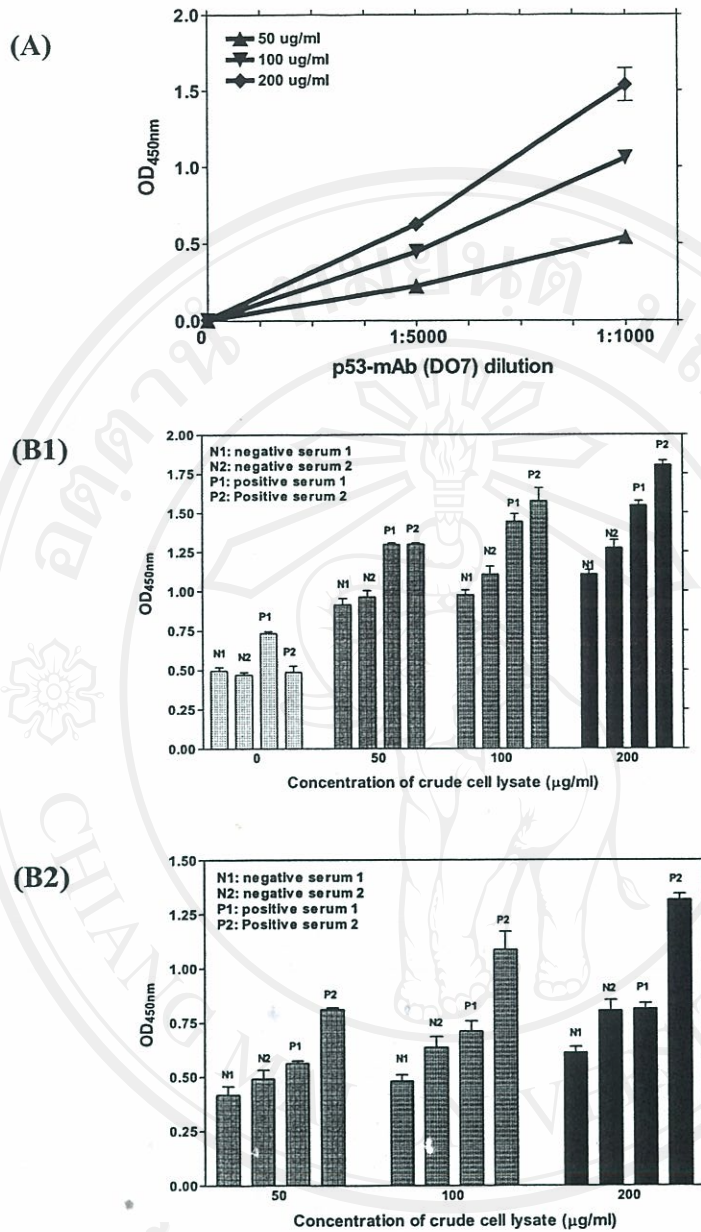


Figure 22. Optimization of the concentration of biotinylated p53-BCCP fusion protein antigens to be coated onto an ELISA plate. **(A)** The biotinylated p53-BCCP fusion protein at 0, 50, 100 and 200 µg/ml were tested for reactivity with anti-p53 monoclonal antibody DO7 at dilution of 0, 1:5000 and 1:1000, respectively. Each point represents the mean value of three determinations. **(B)** Reactivity of serum from cancer patients to biotinylated p53-BCCP fusion protein selectively immobilized onto avidin coated microtiter plate. Various concentration of cell lysate were tested for reactivity with two negative sera and two positive sera at 1/200 dilutions, respectively. **(B1)** total reactivity **(B2)** after subtracting non-specific reactivity with the empty plate. Each bar represents the mean value of two determinations.

4.2 Optimization of ELISA using (His)₆-p53 as antigens

4.2.1 Optimization of (His)₆-p53 concentration

To optimize His₆-p53 protein concentration to be coated onto a microtiter plate, the commercial mouse anti-p53 antibody (DO7) at 0, 1:5000, 1:1000 and 1:500 dilutions and test sera (2 positive and 2 negative proven by western blot) at 1:200 dilution were tested for the presence of p53-specific antibodies with ELISA using various concentrations of the purified His₆-p53 recombinant protein (antigen) (0, 1, 3 and 5 µg/ml). The results presented in Figure 23A show that the commercial p53 antibody did not demonstrate insignificant difference in OD₄₅₀ reading when the antigen coating concentration was altered. For the test sera, in contrast, the antigen at concentration of 5 µg/ml showed the highest OD₄₅₀ values and the best differentiation power to differentiate positive sera from those negative. Therefore, the coating concentration of an antigen for anti p53 antibody detection was set as 5 µg/ml (Figure 23B).

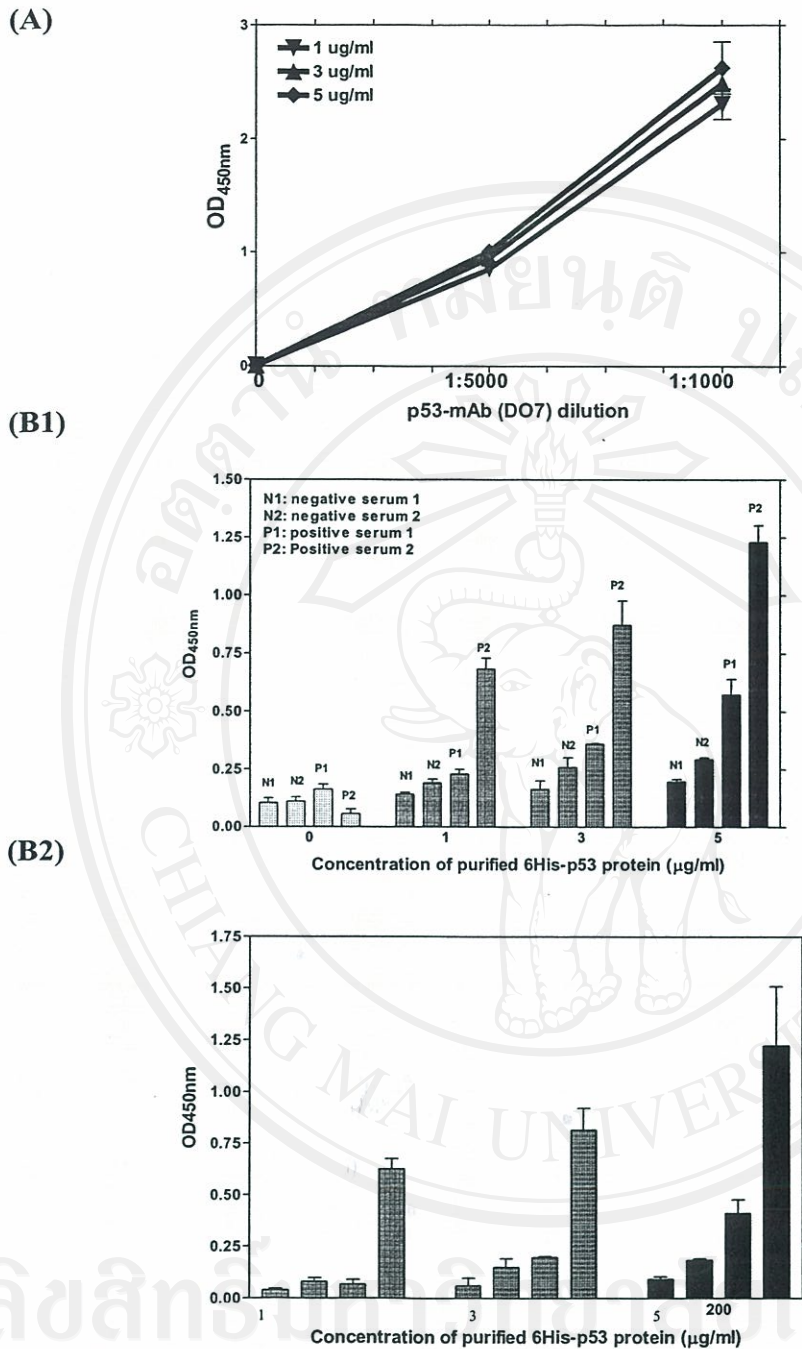
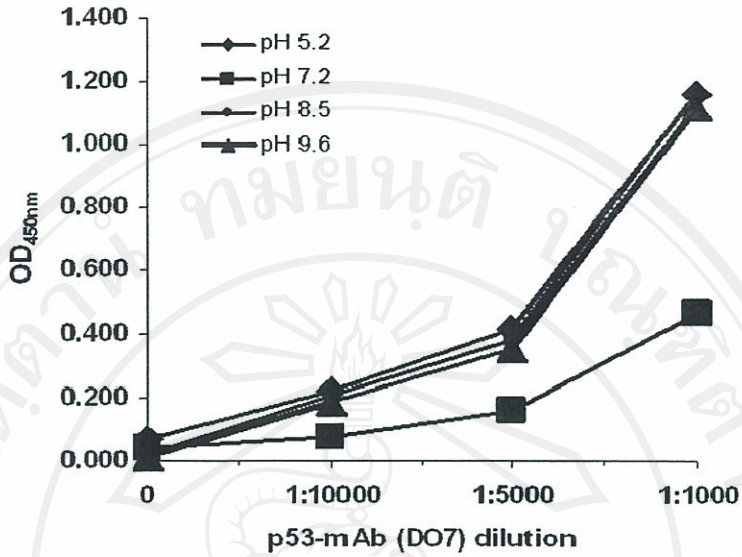


Figure 23. (A) Optimization of the concentration of His₆-p53 antigens to be coated onto an ELISA plate. The His₆-p53 recombinant proteins at 0, 1, 3 and 5 $\mu\text{g/ml}$ were tested for reactivity with anti-p53 monoclonal antibody DO7 at dilution of 0, 1:5000 and 1:1000, respectively. Each point represents the mean value of three determinations. (B) The His₆-p53 recombinant proteins were tested for reactivity with two negative sera and two positive sera for producing p53 autoantibody proven by Western blot analysis at 1:200 dilutions, respectively. (B1) total reactivity (B2) after subtracting non-specific reactivity with the empty plate. Each bar represents the mean value of three determinations.

4.2.2 Optimization of coating buffer for coating (His)₆-p53 onto a microtiter plate

The optimal pH for coating (His)₆-p53 proteins was determined through this experiment. Five µg/ml of (His)₆-p53 proteins were diluted in four different coating buffers (PBS pH 5.2, PBS pH 7.4, Tris pH 8.5 and Carbonate pH 9.6). The commercial mouse anti-p53 antibody (DO7) at 0, 1:5000, 1:1000 and 1:500 dilutions and test sera (2 positive and 2 negative proven by Western blot) at 1:200 dilution were tested for the present of p53-specific antibodies with ELISA using different coating buffer. The result is shown in Figure 24. It was found that reactivity of the commercial p53 antibody (DO7) to (His)₆-p53 was similar except for those using PBS pH7.2, which the reactivity appeared to be less. In test sera, although the reactivity was very similar, (His)₆-p53 antigen coated with carbonate buffer pH 9.6 was the best in differentiating positive sera from negative sera for p53 autoantibody production (Figure 24B).

(A)



(B)

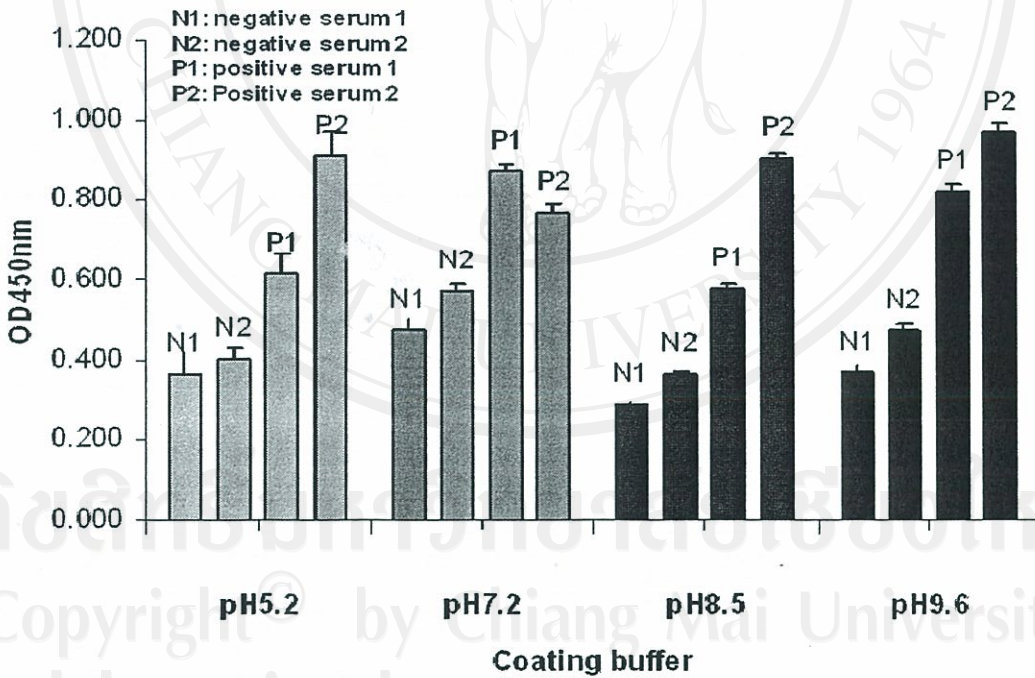


Figure 24. Finding the most suitable buffer for coating (His)₆-p53 onto microtiter plate. His₆-p53 recombinant proteins were diluted in various buffers (PBS pH 5.2, PBS pH 7.4, Tris pH 8.5 and Carbonate pH 9.6). The commercial mouse anti-p53 antibody (DO7) at 0, 1:5000, 1:1000 and 1:500 dilutions (A) and two negative sera and two positive sera at 1/200 dilutions test sera (B) were tested for the present of p53-specific antibodies with ELISA. Each bar or point represents the mean value of three determinations.

5. Evaluation of the established ELISA

5.1 Reproducibility study of the established ELISA

After obtaining the optimal ELISA condition, reproducibility study was done on a pooled serum. Twenty replicates were performed using (His)₆-p53 protein as antigen. As shown in Figure 25, the pooled serum has a mean OD₄₅₀ value of 0.230. The standard deviation was ± 0.022 . This represents the Optimal Condition of variation (OCV) of 9.5, which is acceptable for ELISA.

This pooled control serum was later subjected to ELISA experiment along with serum from cancer patients. Result interpretation of the unknown serum was made only when the mean OD₄₅₀ value of the control serum in triplicate assayed at the same time with unknown serum fell within $0.230 \pm 4(0.022)$ which is between 0.142-0.318.

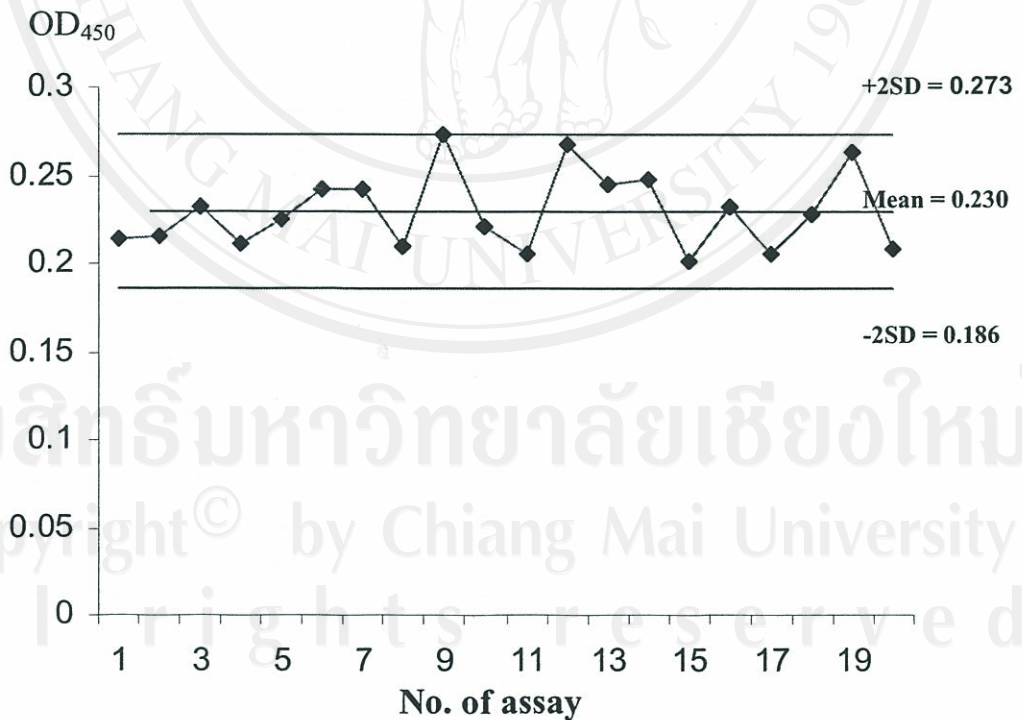


Figure 25. The quality control chart of OD of the control serum by established ELISA. The chart demonstrated the variation of Intra-assay (mean=0.230, SD=0.022).

5.2 Detection of p53 autoantibody in lung cancer patient serum using the generated (His)₆-p53 protein.

The reactivity of antibodies from lung cancer patient sera with the produced (His)₆-p53 was examined by using ELISA. Twenty six sera from lung cancer patient sera from Maharaj Nakorn Chiang Mai Hospital were analyzed to investigate the reactivity assay by ELISA in comparison to Western blot results.

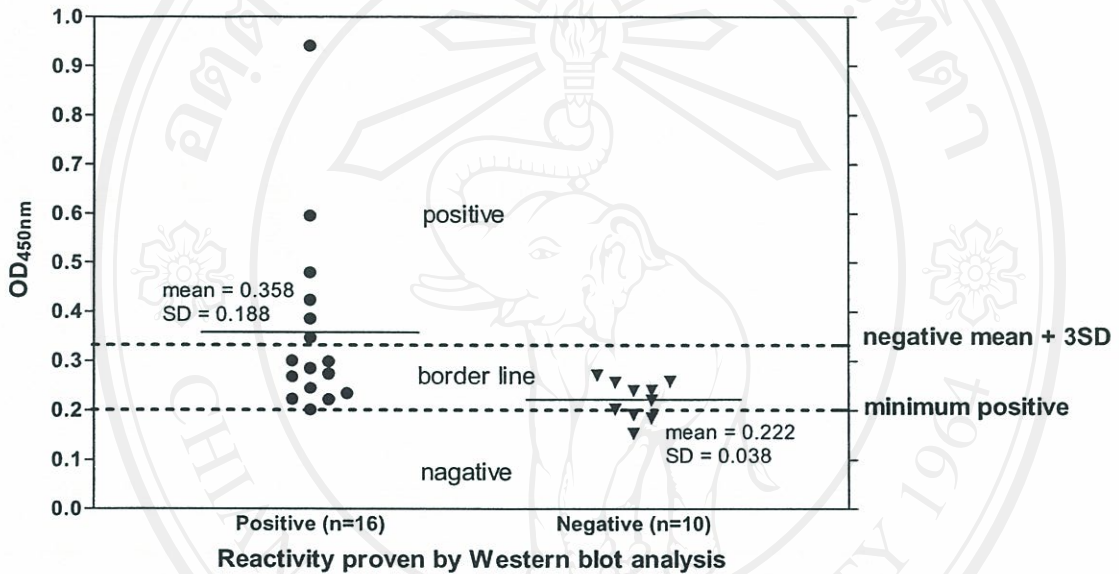


Figure 26 Detection of antibody responses of p53 by established ELISA. Twenty six serum samples from lung cancer patients were analyzed for reactivity with immobilized (His)₆-p53 recombinant protein using the optimized conditions as described in the text.