CHAPTER V

RESULTS

1. Detection of expression level of livin and survivin in tumor tissues and normal tissues with Western blot analysis

In order to detect the expression of livin and survivin proteins, SDS-PAGE and Western blot analysis were performed with cell lysate of tumor tissues in comparison with the corresponding normal tissues of lung cancer patients. The results showed an increasing level of livin and survivin expression in tumor tissues, but not in normal tissues. From tumor tissues, livin was detected in 62 of 67 (93.0%) and survivin was detected in 65 of 67 (97.0%), the representative of immunoblotting results were shown in Figure 1. Livin protein had an estimated molecular weight of 36 kDa and survivin had an estimated molecular weight of 16.5 kDa. Furthermore, the PVDF membrane was stained with Ponceau S in order to examine the amount of total protein loaded into each lane.

We next investigated the clinicopathologic features (sex, age, smoking history, stage of disease and metastasis) of lung tumor samples (n=52) that expressed livin or survivin protein. We found that, no significant correlations were identified against any parameter investigated. The data were shown in Table 1.

Table 1 Clinicopathologic features of lung tumor samples investigated in this study (total number of cases; n = 52)

Clinicopathology	Western blot analysis			
	Livin (48/52)		Survivin (50/52)	
	n (positive/total)	*p*-Value *	n (positive/total)	*p*-Value *
Sex				
Female	18/20	0.622	20/20	0.254
Male	30/32		30/32	
Age				
< 50 years	9/10	0.761	9/10	0.260
≥ 50 years	39/42		41/42	
Smoking history				
Yes	43/47	0.497	45/47	0.638
No	5/5		5/5	
Stage of disease				
I	14/15	0.996	15/15	0.452
II	11/12		11/12	
III	10/11		10/11	
IV	13/14		14/14	
Metastasis				
No	35/38	0.928	36/38	0.381
Yes	13/14		14/14	

*** Chi-square test**

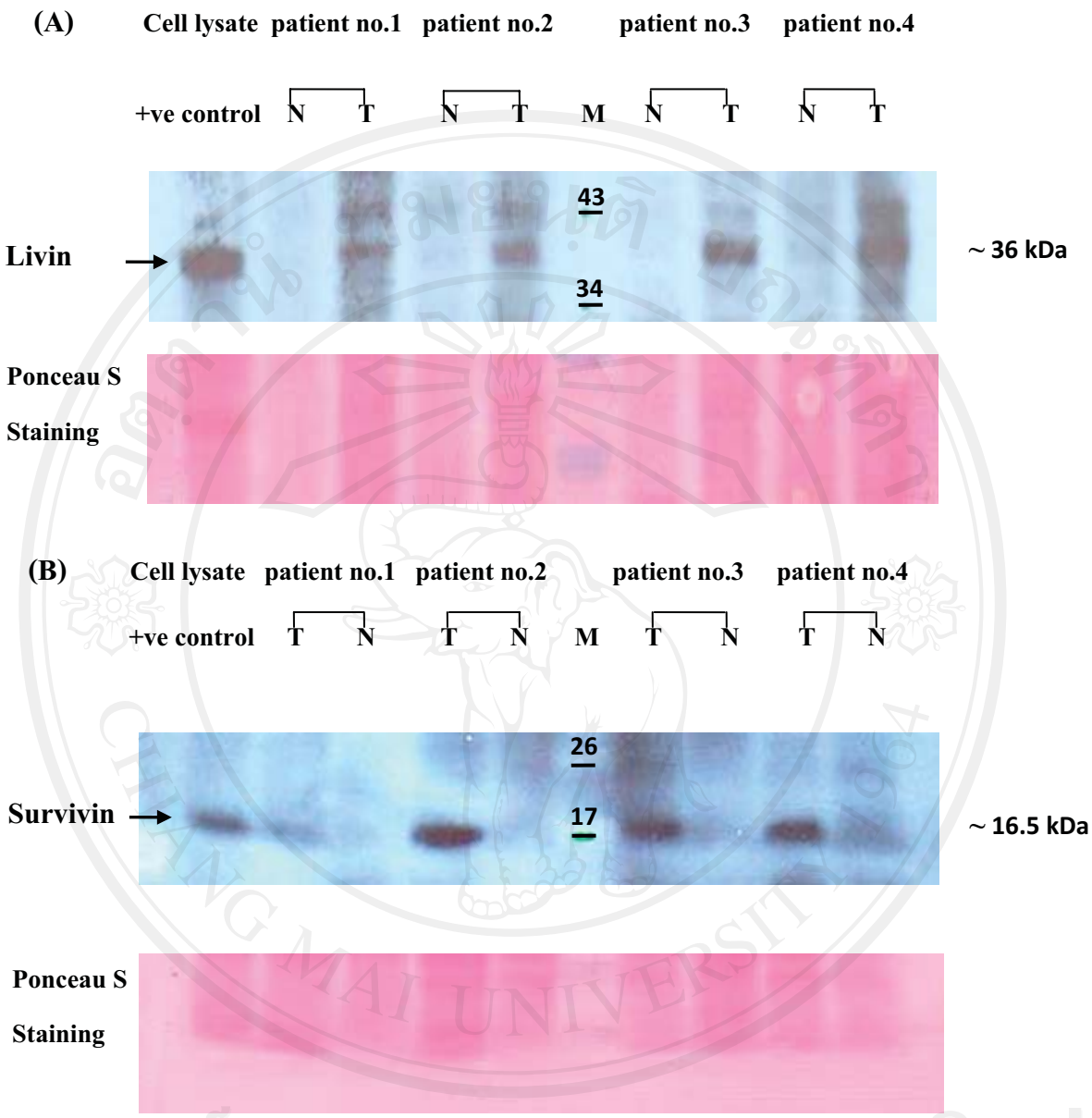


Figure 1. Representative western blot analysis of cell lysates (T = tumor tissue and N = normal tissue) from lung cancer patients. 30 µg of cell lysates were electrophoresed and electrobloted onto PVDF membrane. The PVDF membrane was detected with anti-livin mAb. The expression of livin protein existing in cell lysates of tumor tissues recognized an approximately 36 kDa protein in positive control and in tumor tissues (A). Also, the PVDF membrane was detected with anti-survivin mAb. The expression of survivin protein existing in cell lysates of tumor tissues recognized an approximately 16.5 kDa protein in positive control and in tumor tissues (B).

2. Detection of autoantibodies against livin and survivin in lung cancer patients

2.1 Production of recombinant livin and survivin tumor antigens

2.1.1 Construction of expression vectors (pET-15b-livin and pET-15b-survivin)

In order to amplify the full-length livin and survivin encoding DNAs from HeLa cells, RT-PCRs were performed using a set of primer including NdeIlivin and NdeIsurvivin primers carrying restriction sequence for *NdeI* at 5' terminal in combination with livinBamHI and survivinBamHI primers possessing restriction sequence for *BamHI* at 3' terminal, respectively. From this experiment, the NdeI-livin-BamHI and NdeI-survivin-BamHI DNA fragments which carrying the restriction site for *NdeI* and *BamHI* at the terminus of livin and survivin encoding genes were generated. To confirm the PCR amplification, the PCR product was fractionated using 1.5% agarose gel electrophoresis. Successful amplification of the NdeI-livin-BamHI and NdeI-survivin-BamHI were clearly indicated by the presence of a band of approximately 1300 bp (Figure 2) and 400 bp (Figure 3), respectively. After that, NdeI-livin-BamHI and NdeI-survivin-BamHI PCR products were purified using PCR purification kit and followed by double digested with *NdeI* and *BamHI* to produce sticky ends. Meanwhile, pET-15b vectors were double digested with *NdeI* and *BamHI* to linearize the supercoiled plasmid. After obtaining the purified DNA fragments and vectors, the digested NdeI-livin-BamHI and NdeI-survivin-BamHI DNA fragments were then inserted and ligated into the *NdeI* and *BamHI* treated pET-15b vectors. To confirm the appropriate ligation, the ligation products were verified using T7 promoter and T7 terminator primers. Successful ligation were confirmed by the presence of PCR products in lane 1 of Figure 4 and 5. The higher molecular weight bands presented in lane 1 indicated that livin or

survivin encoding DNA was inserted into pET-15b vector. Two strong bands presented in this lane with 250 and 1400 or 250 and 700 bp in size are believe to be the amplified products from self ligated pET-15b vector and vector with livin or survivin gene inserted, respectively. Taken all together, the result in Figure 4 and 5 demonstrated that the desired ligation products had occurred. After ensuring the appropriate ligation, the mixture was then use to transform *E.coli* strain XL-1blue cells. The ampicillin resistant colonies were selected and screened for the pET-15b-livin and pET-15b-survivin transformed clones. The resistant colonies were then subjected to PCR with the same pair of primers used to verify the ligation products (T7 promoter and T7 terminator primers). One out of three clones was found to have up-taken pET-15b-livin construct by the present of the expected size of PCR products (~1400 bp) and 4 clones were found to have up-taken pET-15b-survivin construct by the present of the expected size of PCR products (~700 bp), as show in Figure 6 and 7. To confirm the PCR results, plasmids were extracted from these positive clones and subjected to digestion with the same pair of restriction enzymes (*NdeI* and *BamHI*) used to generate sticky end before the ligation process. The same DNA constructs were also sent to First base, Singapore in order to perform DNA sequencing analysis. In Figure 8, the present of DNA band at molecular weight around 6000 bp of the remaining pET-15b vector and 1300 bp of the livin inserted DNA after digestion confirmed that the transformant have up taken the pET-15b-livin DNA construct. Mean while, in Figure 9, the present of DNA band at molecular weight around 6000 bp of the remaining pET-15b vector and 400 bp of the survivin inserted DNA after digestion confirmed that the transformant have up taken the pET-15b-survivin DNA construct. Furthermore, the DNA sequencing

resulted from the First base confirmed that both of the obtained clones carried the corrected pET-15b-livin DNA construct, isoform α and pET-15b-survivin DNA construct, isoform 2B. Therefore, both of the DNA constructs were further used to produce recombinant proteins.

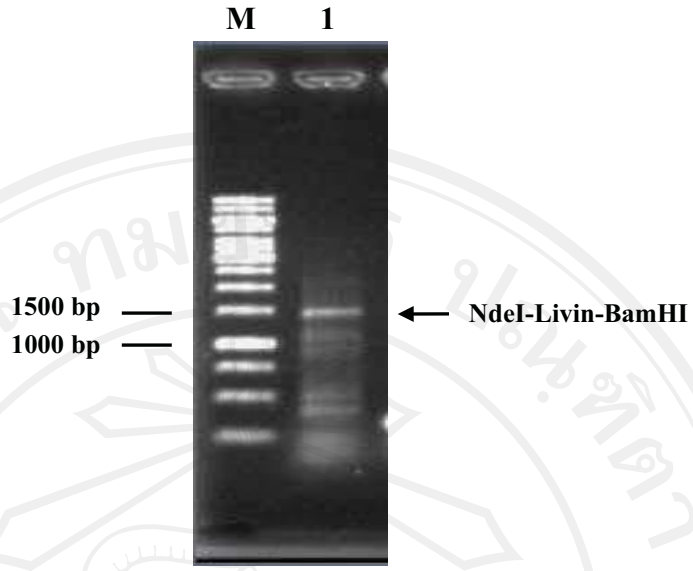


Figure 2. Agarose gel analysis showing the obtained livin PCR products using NdeIlivin and livinBamHI as primers which was referred as "NdeI-livin-BamHI".
Lane M = DNA molecular weight marker and Lane 1 = NdeI-livin-BamHI.

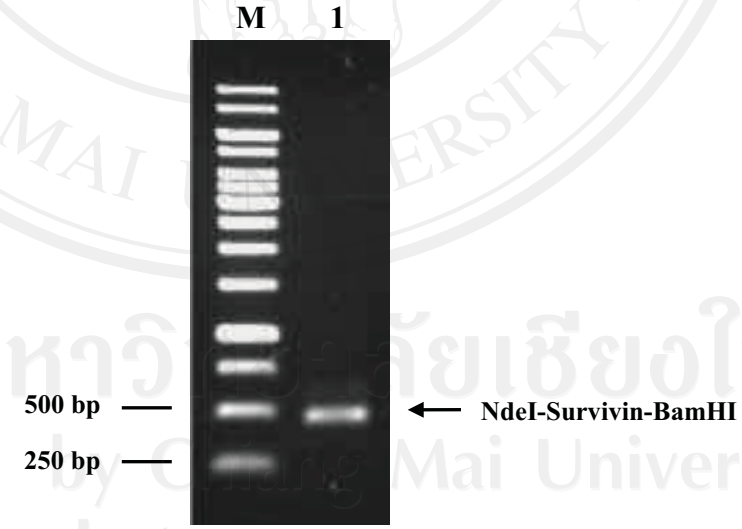


Figure 3. Agarose gel analysis showing the obtained survivin PCR products using NdeIsurvivin and survivinBamHI as primers which was referred as "NdeI-survivin-BamHI". Lane M = DNA molecular weight marker and Lane 1 = NdeI-survivin-BamHI.

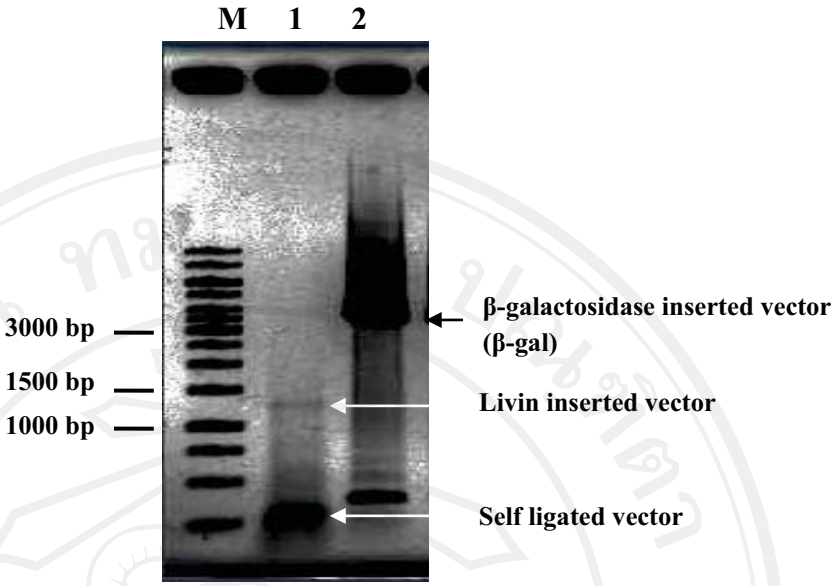


Figure 4. An example of agarose gel analysis of PCR products to verify the appropriate ligation between pET-15b vector and the inserted DNA in ligation mixture.
Lane M = DNA molecular weight marker, Lane 1 = Amplified NdeI-livin-BamHI PCR product using T7 promoter and T7 terminator primers and Lane 2 = Ligation control of pET-15b vector and β-galactosidase (β-gal)

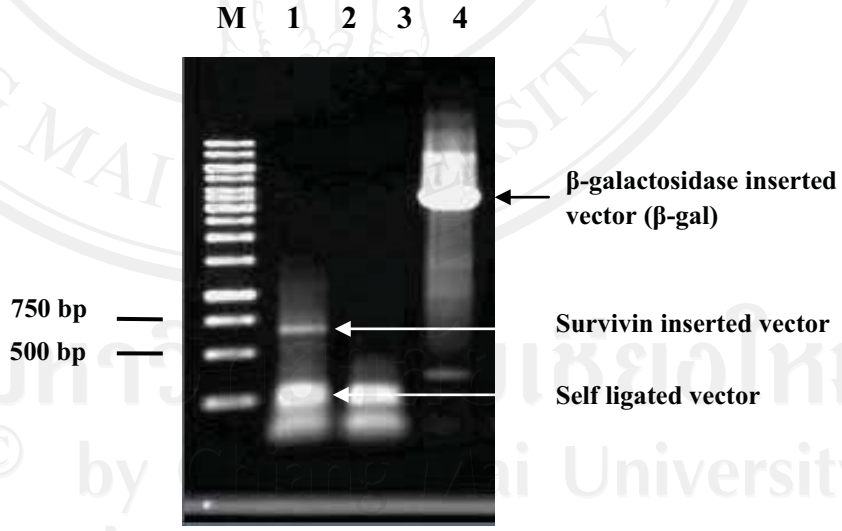


Figure 5. An example of agarose gel analysis of PCR products to verify the appropriate ligation between pET-15b vector and the inserted DNA in ligation mixture. Lane M = DNA molecular weight marker, Lane 1 = Amplified NdeI-survivin-BamHI PCR product using T7 promoter and T7 terminator primers, Lane 2 = Self ligated vector and Lane 3 = Ligation control of pET-15b vector and β-galactosidase (β-gal)

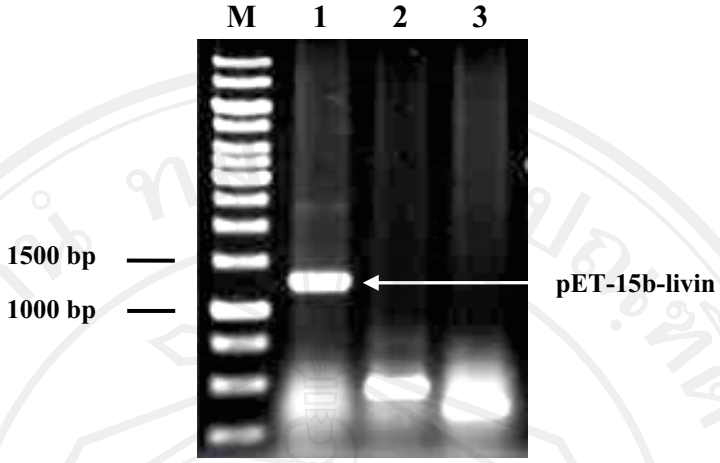


Figure 6. Agarose gel analysis of antibiotic resistant colonies screening for pET-15b-livin vector uptaking clone by PCR.
Lane M = DNA molecular weight marker and Lane 1-3 = amplified PCR product from ampicillin resistant clone using T7 promoter and T7 terminator primers

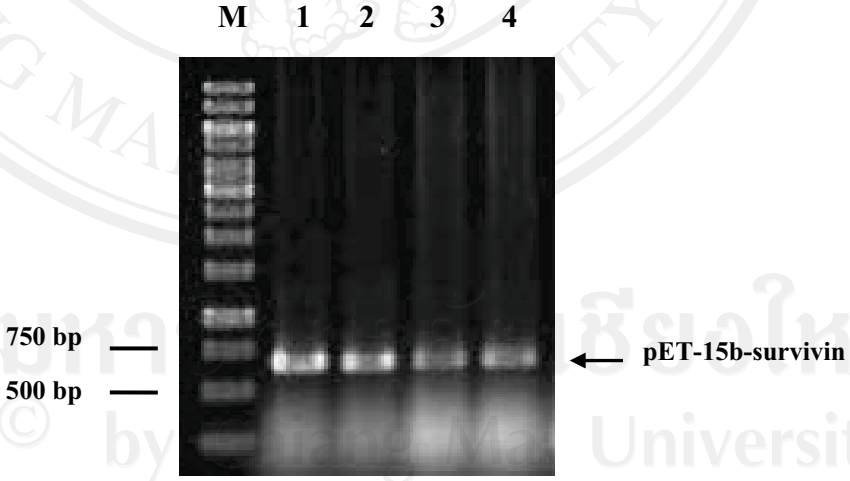


Figure 7. Agarose gel analysis of antibiotic resistant colonies screening for pET-15b-survivin vector uptaking clone by PCR.
Lane M = DNA molecular weight marker and Lane 1-4 = amplified PCR product from ampicillin resistant clone using T7 promoter and T7 terminator primers

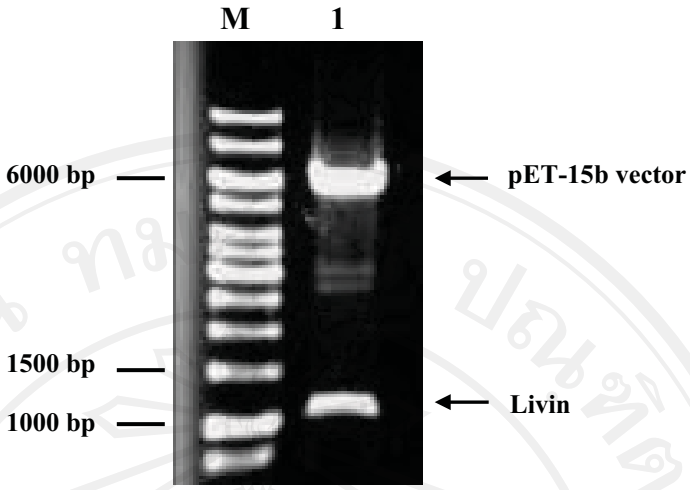


Figure 8. Agarose gel analysis of restriction enzyme digested products to confirm the obtained positive colony.
Lane M = DNA molecular weight marker and Lane 1 = *NdeI* and *BamHI* digested Pet-15b-livin plasmid

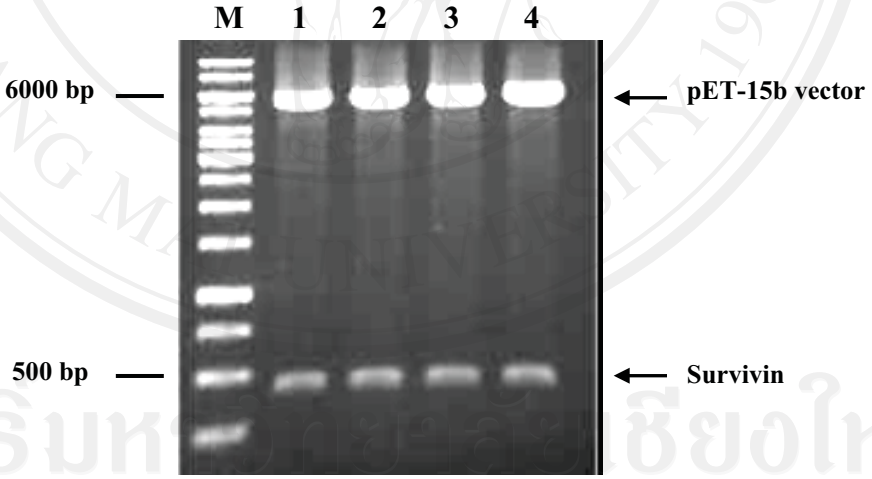


Figure 9. Agarose gel analysis of restriction enzyme digested products to confirm the obtained positive colonies.
Lane M = DNA molecular weight marker and Lane 1-4 = *NdeI* and *BamHI* digested pET-15b-survivin plasmid

2.1.2 Expression of livin and survivin recombinant proteins in bacterial host cells

The recombinant pET-15b-livin and pET-15b-survivin DNA constructs were 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 sequence encoding six histidine residues at 5' of the multiple cloning sites (MCS). The hexahistidine-livin and hexahistidine-survivin fusion proteins would have an estimated size of about 48 kDa and 25 kDa, respectively, on SDS-PAGE. The results from Western blotting analysis showed that basal expression of livin and survivin were minimal without IPTG induction both in BL21(DE3) and BL21(DE3)pLysS. On the other hand, IPTG induction greatly increased level of the expression since 48 kDa of livin and 25 kDa of survivin were detected (Figure 10-13). Therefore, the Western blotting results confirmed that livin and survivin proteins were successfully expressed in *E.coli* BL21(DE3) and BL21(DE3)pLysS. Nevertheless, *E.coli* BL21(DE3)pLysS was chosen to be an expression host cells to produce livin and survivin proteins as their strain of bacteria produced higher level of recombinant proteins in comparison to *E.coli* BL21(DE3).

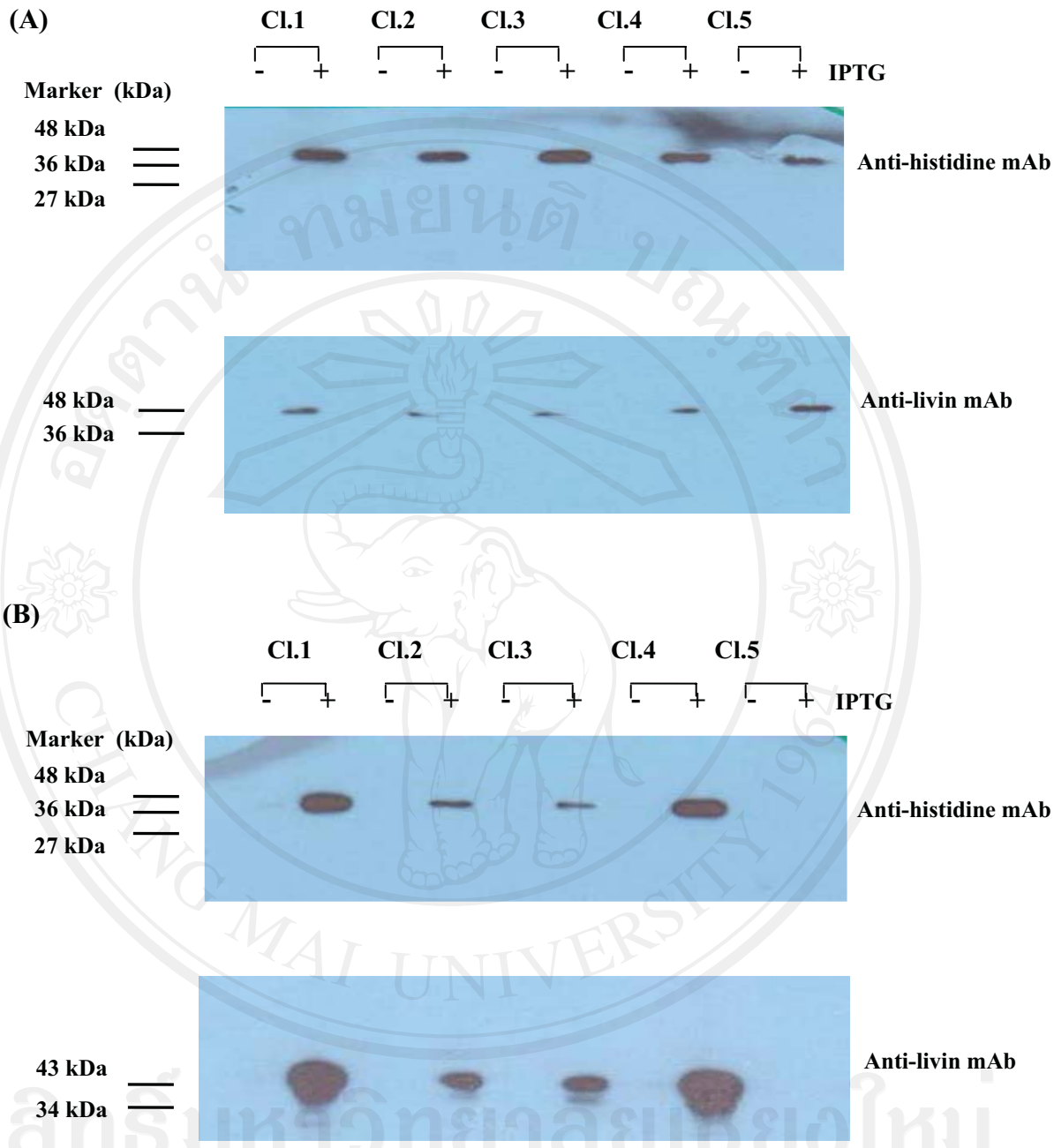


Figure 10. Western blot analysis of hexahistidine-livin fusion protein expressed in *E.coli* DE3 (A) and (DE3)pLysS (B) cells using anti-histidine mAb and anti-livin mAb. 2 µg of cell lysates were electrophoresed and electrobloted onto PVDF membranes. The PVDF membranes were incubated with anti-histidine mAb and anti-livin mAb. The expression of livin protein existing in cell lysates recognized an approximately 48 kDa protein in IPTG induction clones, Cl.1 (+), Cl.2 (+), Cl.3 (+), Cl.4 (+) and Cl.5 (+).

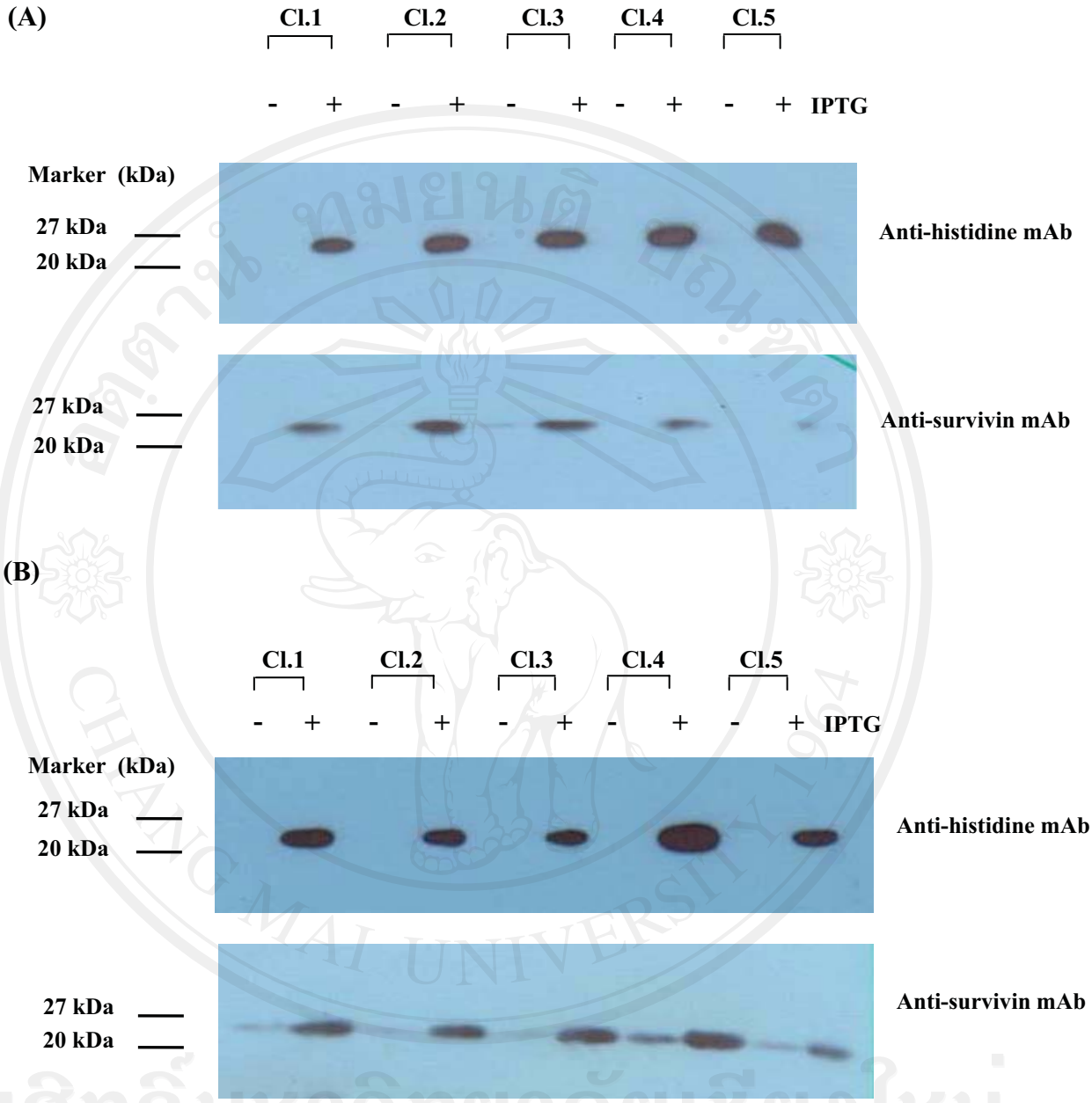


Figure 11. Western blot analysis of hexahistidine-survivin fusion protein expressed in *E.coli* DE3 (A) and DE3pLysS (B) cells using anti-histidine mAb and anti-survivin mAb. 2 µg of cell lysates were electrophoresed and electrobloted onto PVDF membranes. The PVDF membranes were incubated with anti-histidine mAb and anti-survivin mAb. The expression of survivin protein existing in cell lysates recognized an approximately 25 kDa protein in IPTG induction clones, Cl.1(+), Cl.2(+), Cl.3(+), Cl.4(+) and Cl.5(+).

2.2 Optimization of ELISA

2.2.1 Optimization of antigen concentration

To optimize $(His)_6$-livin and $(His)_6$-survivin protein concentration to be coated onto a microtiter plate, the commercial antibodies at various dilutions and test sera (2 positive and 2 negative) at 1:1000 dilution were tested for the presence of livin and survivin specific autoantibodies with ELISA using various concentrations of the crude $(His)_6$-livin recombinant protein (antigen) (0, 25, 50, 100 and 200 µg/ml). The results represent in Figure 12, showed that anti-livin mAb had a similar in OD_{450} reading when the antigen coating concentration was altered. For the test sera, the antigen at concentration of 100 µg/ml showed the suitable OD_{450} values and the differentiation power to discriminate positive sera (patient sera) from those negative (normal sera). Therefore, the coating concentration of an antigen for livin autoantibody detection was set as 100 µg/ml (Figure 12). For survivin tumor antigen, the results represent in Figure 13, showed that anti-survivin mAb had a significant difference in OD_{450} reading when the antigen coating concentration was altered. For the test sera, the antigen at concentration of 25 µg/ml showed the suitable OD_{450} values and the well differentiation power to differentiate positive sera from those negative. Therefore, the coating concentration of antigen for survivin autoantibody detection was set as 25 µg/ml (Figure 13).

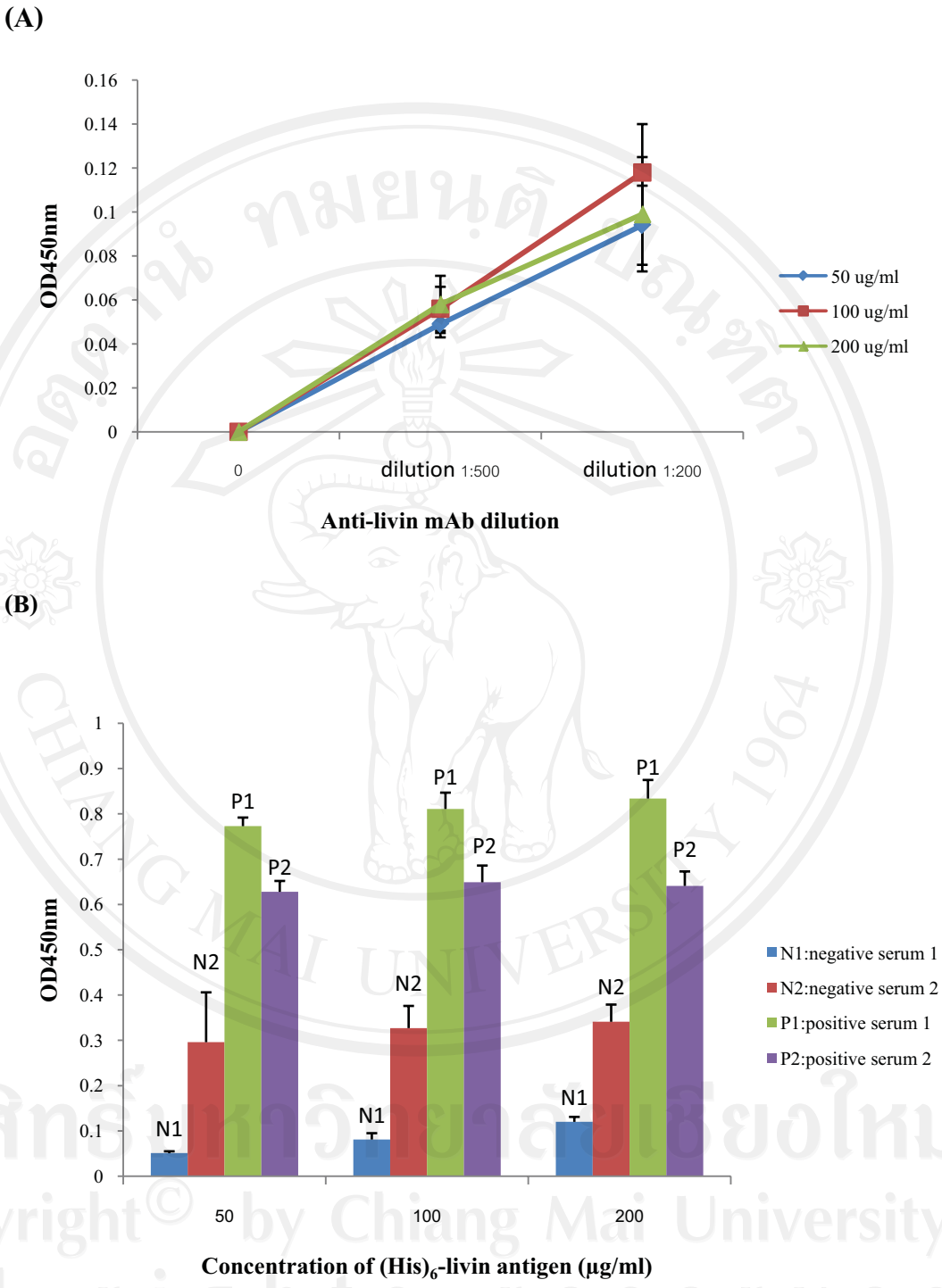


Figure 12. Optimization of the concentration of His_6-livin antigen to be coated onto an ELISA plate. The His_6-livin recombinant protein at 0, 50, 100 and 200 µg/ml were tested for reactivity with anti-livin mAb at dilution of 1:500 and 1:200, respectively. Each point represents the mean value of two determinations (A). The His_6-livin recombinant protein was tested for reactivity with two negative sera and two positive sera for producing livin autoantibody at 1:1000 dilutions, respectively (B), after abstract non-specific reactivity with the empty plate. Each bar represents the mean value of two determinations.

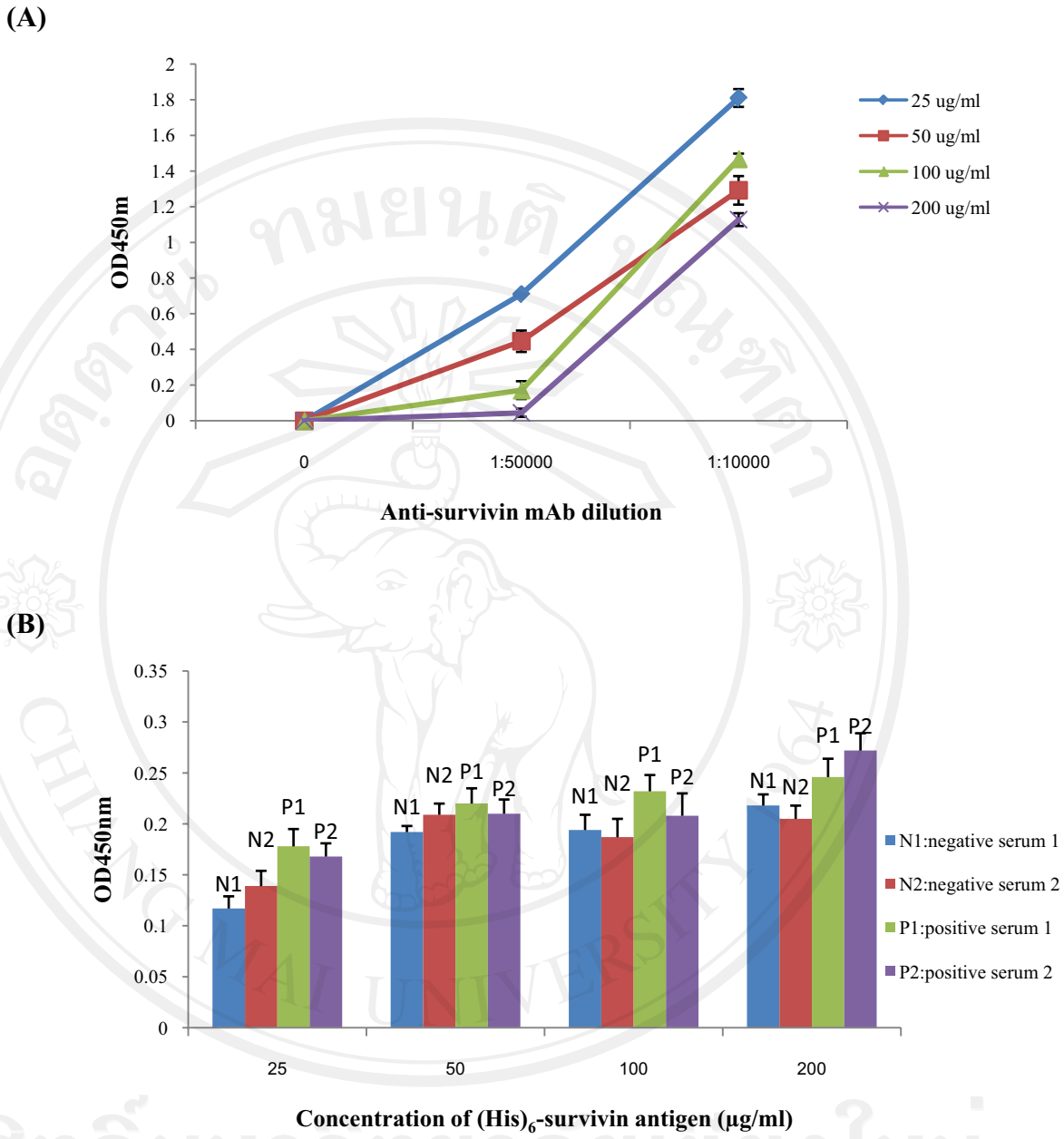


Figure 13. Optimization of the concentration of His_6-survivin antigen to be coated onto an ELISA plate. The His_6-survivin recombinant protein at 0, 25, 50, 100 and 200 µg/ml were tested for reactivity with anti-survivin mAb at dilution of 1:50000 and 1:10000, respectively. Each point represents the mean value of two determinations (A). The His_6-survivin recombinant protein was tested for reactivity with two negative sera and two positive sera for producing survivin autoantibody at 1:1000 dilution, respectively (B), after abstract non-specific reactivity with the empty plate. Each bar represents the mean value of two determinations.

2.2.2 Optimization of coating buffer for coating $(His)_6$-livin and $(His)_6$-survivin antigens onto a microtiter plate

The optimal pH for coating $(His)_6$-livin and $(His)_6$-survivin proteins were determined through this experiment. One hundred μg/ml of $(His)_6$-livin and 25 μg/ml of $(His)_6$-survivin 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). Anti-livin, anti-survivin mAbs and test sera (2 positive and 2 negative) at 1:1000 dilution were tested for the presence of livin and survivin specific autoantibodies with ELISA using different coating buffer. The results showed in Figure 14-15. It was found that reactivity of anti-livin and anti-survivin mAbs to $(His)_6$-livin and $(His)_6$-survivin have a significant difference in OD_{450} reading when the coating buffer was altered. In test sera, although the reactivity was similar, $(His)_6$-livin antigen coated with Tris pH 8.5 was the best in differentiating positive sera from negative sera for livin autoantibody detection (Figure 14). For survivin, $(His)_6$-survivin antigen coated with carbonate pH 9.6 was the best in differentiating positive sera from negative sera for survivin autoantibody detection (Figure 15).

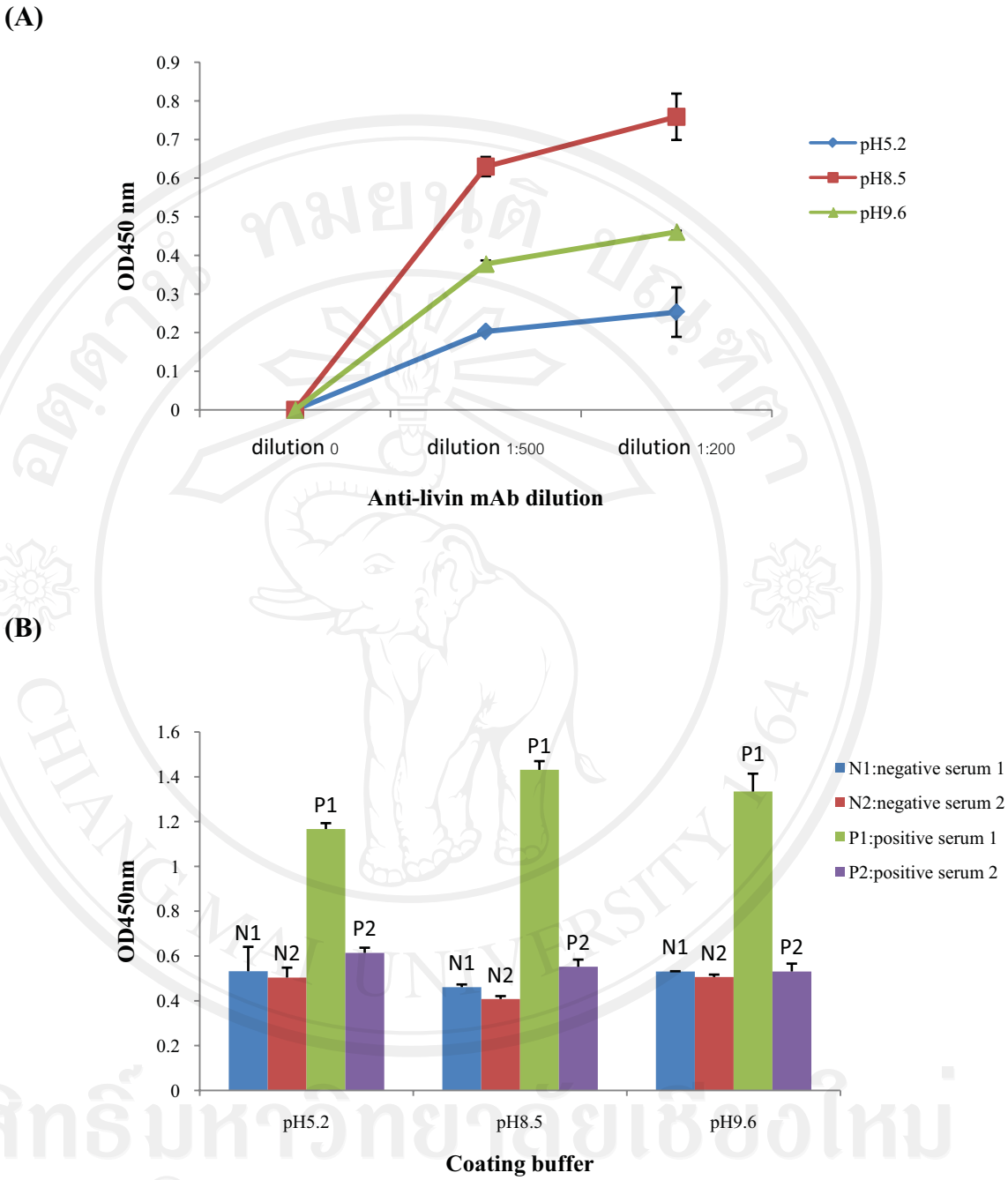
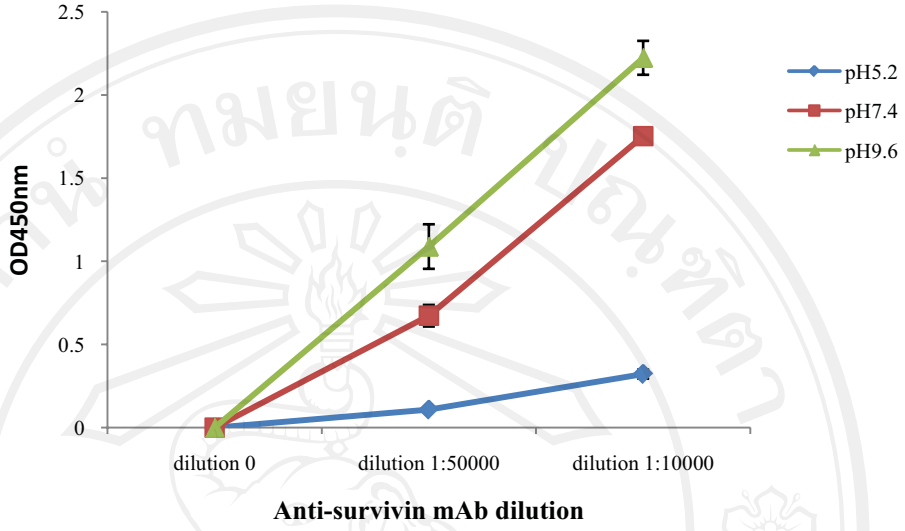


Figure 14. Finding the most suitable buffer for coating $(His)_6$-livin antigen onto microtiter plate. $(His)_6$-livin recombinant protein was diluted in various buffers (PBS pH 5.2, Tris pH 8.5 and carbonate pH 9.6). Anti-livin mAb at 0, 1:500 and 1:200 dilutions (A), two negative sera and two positive sera at dilution of 1:1000 (B) were tested for the present of livin-specific autoantibody with ELISA. Each bar or point represents the mean value of two determinations.

(A)



(B)

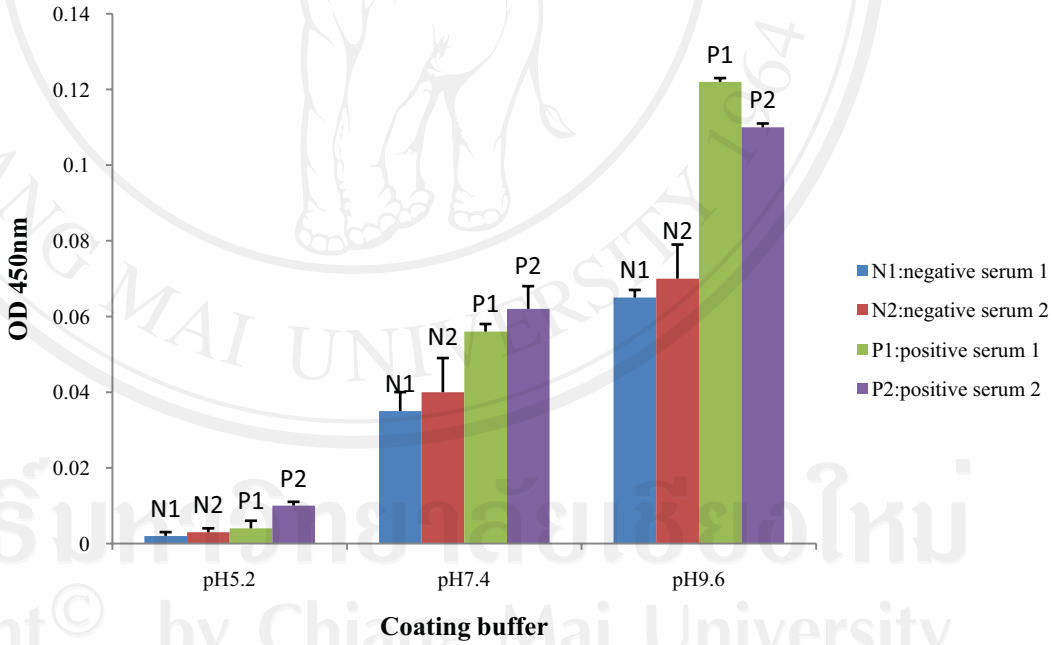


Figure 15. Finding the most suitable buffer for coating $(His)_6$-survivin antigen onto microtiter plate. $(His)_6$-survivin recombinant protein was diluted in various buffers (PBS pH 5.2, PBS pH 7.4 and carbonate pH 9.6). Anti-survivin mAb at 0, 1:50000 and 1:10000 dilutions (A), two negative sera and two positive sera at 1:1000 dilution (B) were tested for the present of survivin-specific autoantibody with ELISA. Each bar or point represents the mean value of two determinations.

2.2.3 Optimization of blocking agent

Blocking agents including various concentrations of BSA and skimmed milk were tested for their ability to correctly differentiate positive sera from negative sera with low non-specific background. The results showed in Figure 16. It was found that reactivity of anti-livin mAb to $(His)_6$-livin recombinant protein, 0.5% BSA was the best in three different blocking agents. For test sera, the plate blocked with 5% skimmed milk was the best in differentiating positive sera from negative sera for livin autoantibody detection (Figure 16); therefore 5% skimmed milk was further used as blocking agent for detection of anti-livin autoantibody. For survivin, the results in Figure 17 showed that reactivity of anti-survivin mAb to $(His)_6$-survivin recombinant protein was similar in four different blocking agent. In test sera, the plate blocked with 0.5% BSA was the best in differentiating positive sera from negative sera for survivin autoantibody detection (Figure 17).

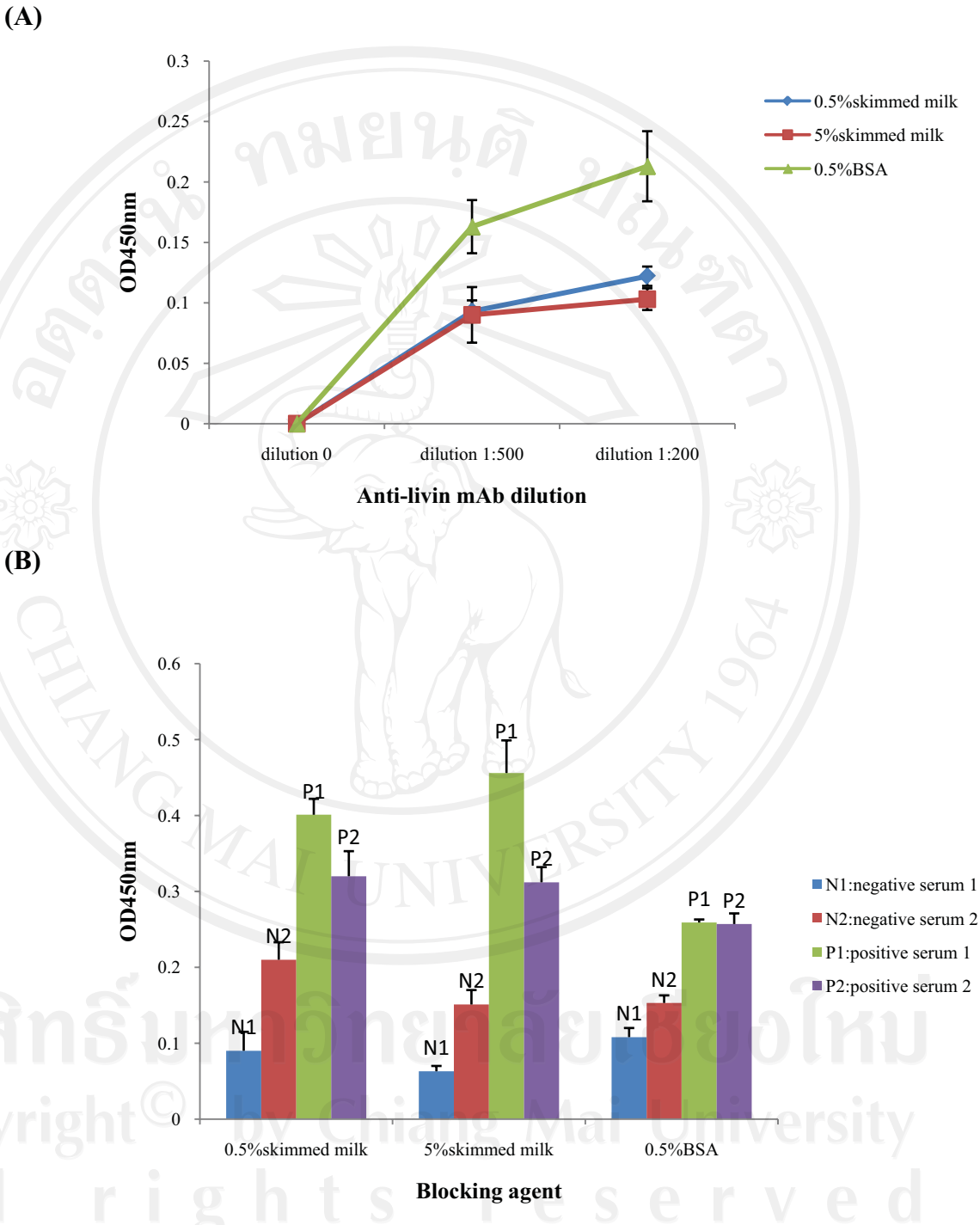


Figure 16. Finding the most suitable blocking agent for detection of anti-livin autoantibody. Various blocking agent (0.5% skimmed milk, 5% skimmed milk and 0.5% BSA) were blocked onto microtiter plate. Anti-livin mAb at 0, 1:500 and 1:200 dilutions (A), two negative sera and two positive sera at 1:1000 dilution (B) were tested for the present of livin-specific autoantibody with ELISA. Each bar or point represents the mean value of two determinations.

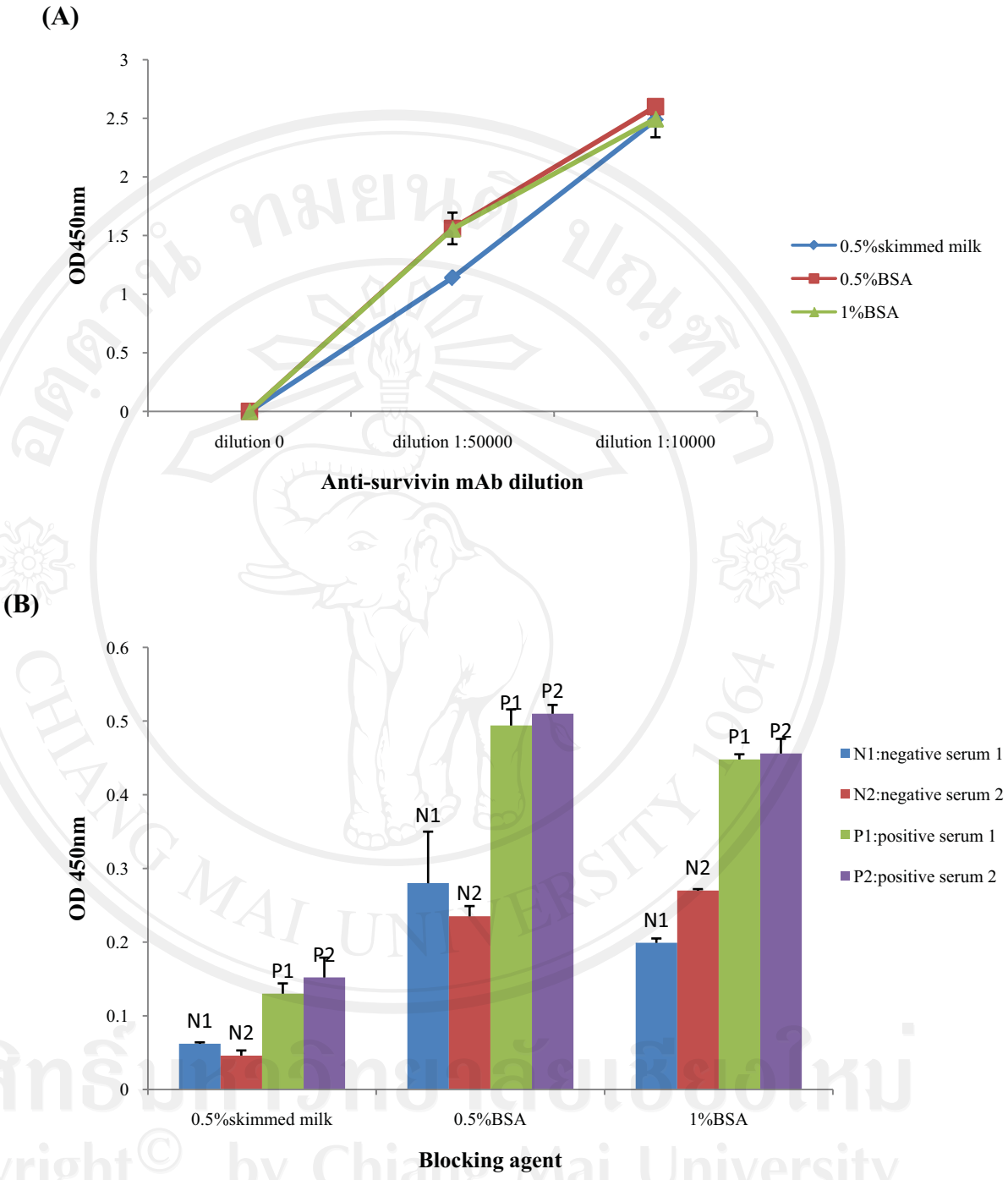


Figure 17. Finding the most suitable blocking agent for detection of anti-survivin autoantibody. Various blocking agent (0.5% skimmed milk, 0.5% BSA and 1% BSA) were blocked onto microtiter plate. Anti-survivin mAb at 0, 1:50000 and 1:10000 dilutions (A), two negative sera and two positive sera at 1:1000 dilution (B) were tested for the present of survivin-specific autoantibody with ELISA. Each bar or point represents the mean value of two determinations.

2.2.4 Optimization of serum dilution

Serum contains a very complex mixture of high titer antibodies; therefore, it is necessary to dilute serum in order to reduce non-specific background from the relevant antibody. For anti-livin autoantibody detection, the test sera were diluted to 1:1000, 1:2500 and 1:5000 with 5% skimmed milk in wash buffer. The results present in Figure 18(A), it was found that reactivity of test sera to $(His)_6$-livin in dilution of 1:1000 was the best in differentiating positive sera from negative sera. For anti-survivin autoantibody detection, test sera (2 positive and 2 negative) with dilution of 1:200, 1:1000 and 1:5000 were diluted in 0.5% BSA in wash buffer were tested. The results showed in Figure 18(B), demonstrated that reactivity of test sera to $(His)_6$-survivin in dilution of 1:1000 was also the best to differentiate positive sera from those that were negative.

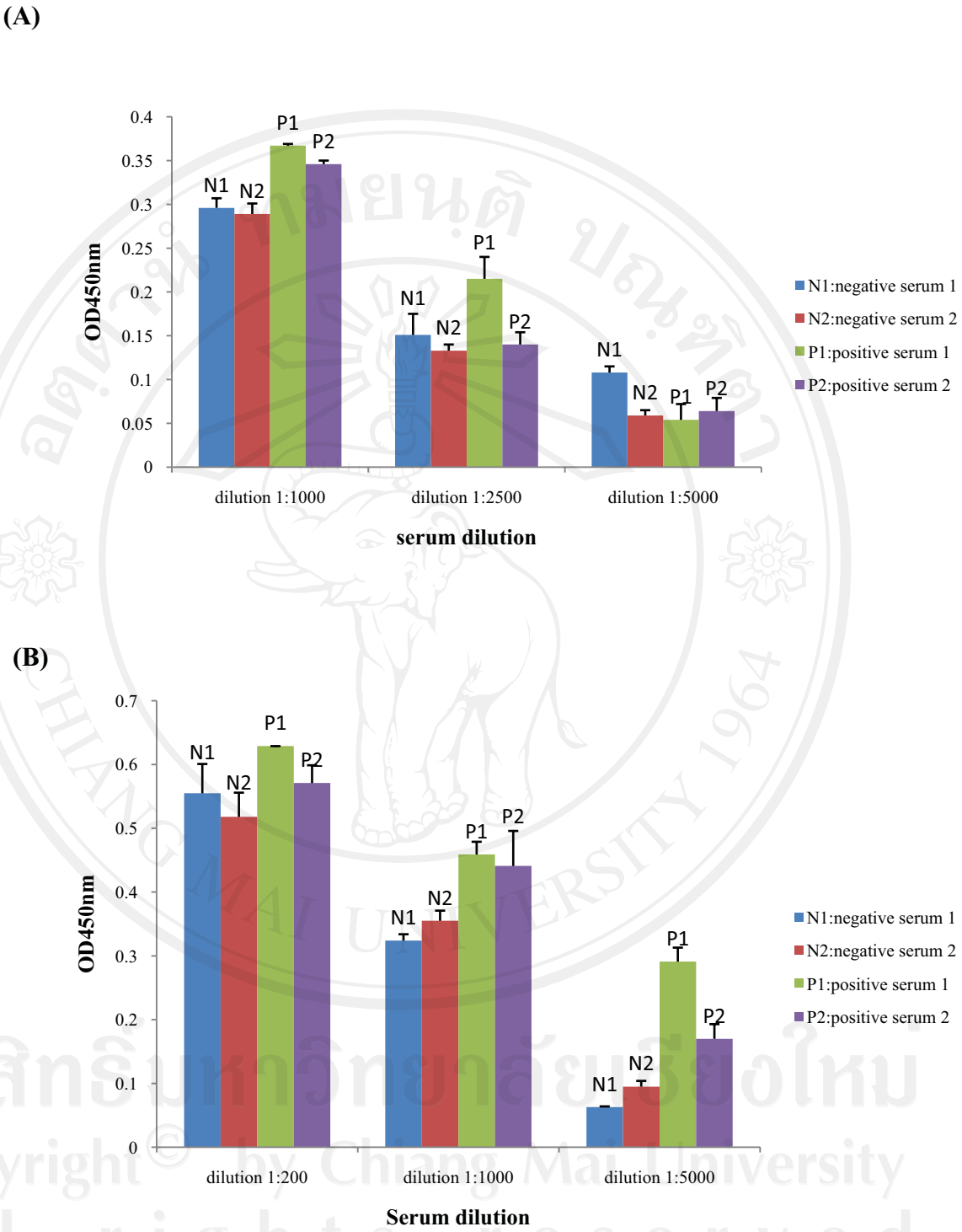


Figure 18. Finding the most suitable serum dilution for detection of anti-livin antibody (A) and anti-survivin antibody (B). Each bar represents the mean value of two determinations.

2.3 Evaluation of the established ELISA

2.3.1 Reproducibility study of the established ELISA

After obtaining the optimal ELISA condition, reproducibility study was done on a positive serum control. Twenty replicates were performed using $(His)_6$-livin and $(His)_6$-survivin proteins as antigens. As shown in Figure 19, for livin, the positive serum control has a mean OD_{450} value of 0.83. The standard deviation was 0.027. This represent % CV of 3.2. In addition, from Figure 20, for survivin the positive serum control has a mean OD_{450} value of 0.85. The standard deviation was 0.044 and the represent % CV of 5.2. Therefore, they are acceptable for ELISA.

This positive serum control was later subjected to ELISA experiment along with serum from lung cancer patients. Result interpretation of the unknown serum was made only when the mean OD_{450} value of the control serum in duplicated assay at the same time with unknown serum for livin fell within 0.83+/- 4(0.027), which is between 0.727-0.938 and for survivin, the result of unknown serum was interpreted when the mean OD_{450} value of the control serum fell within 0.85+/- 4(0.044), which is between 0.674-1.026.

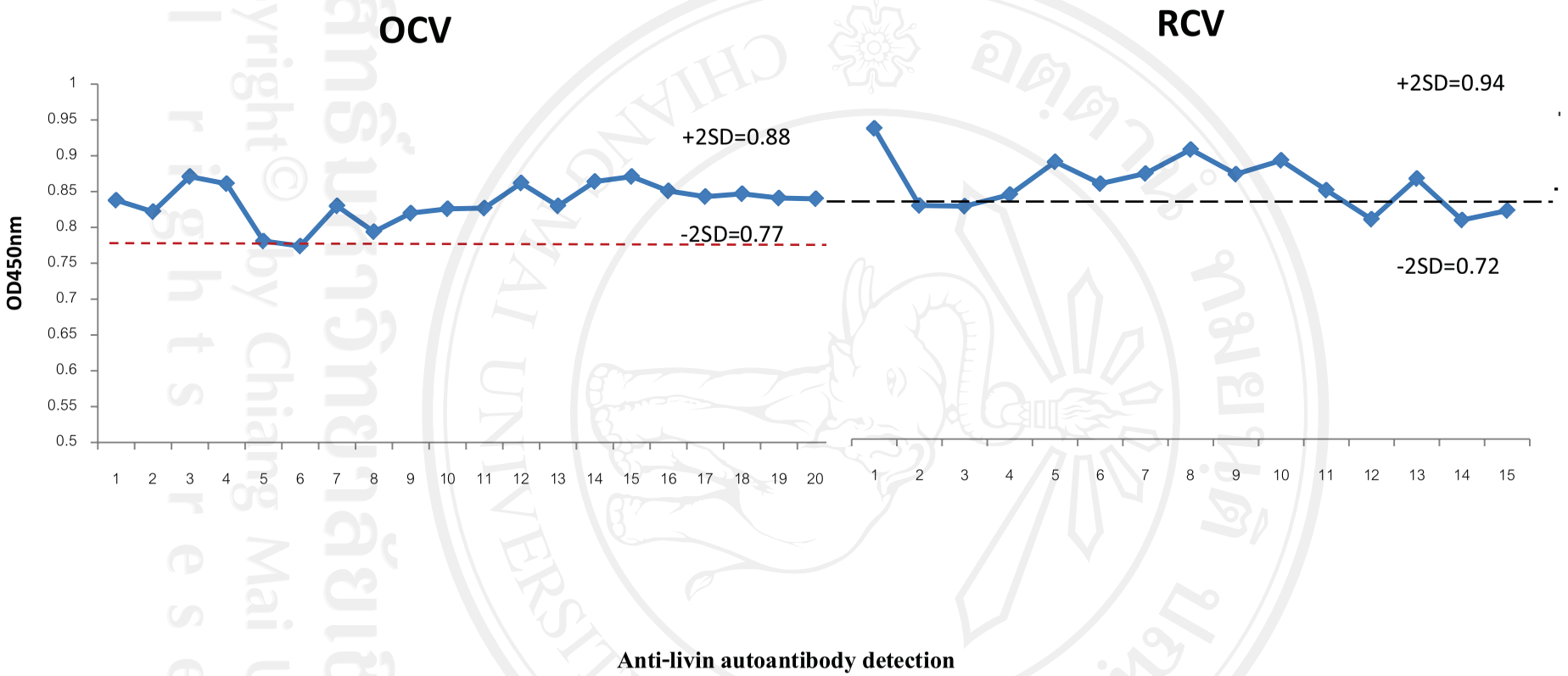


Figure 19. The quality control chart of livin's OD of the control serum by established ELISA. The chart demonstrated the Optimum Condition Variance (OCV; mean=0.83, SD=0.027) and the Routine Condition Variance (RCV; mean=0.85, SD=0.037).

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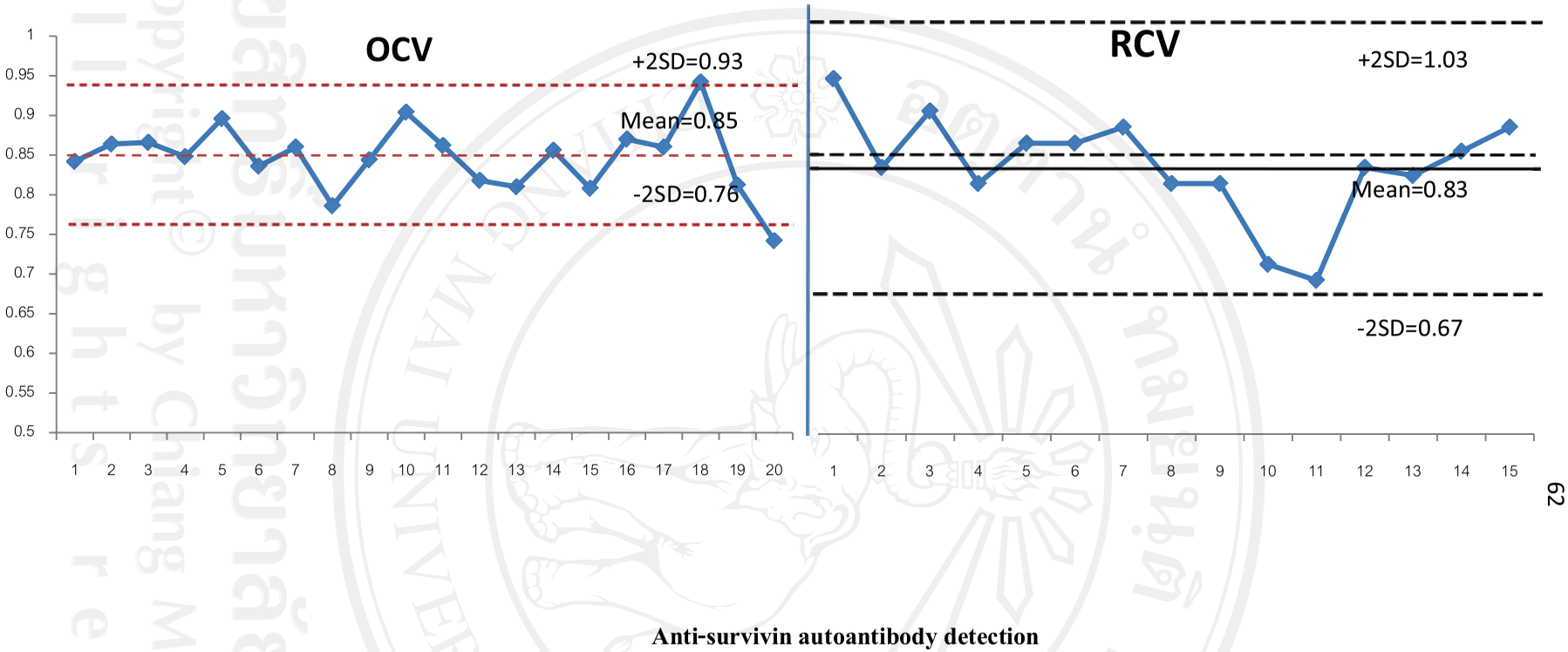

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Figure 20. The quality control chart of survivin's OD of the control serum by established ELISA. The chart demonstrated the Optimum Condition Variance (OCV; mean=0.85, SD=0.044) and the Routine Condition Variance (RCV; mean=0.83, SD=0.065).

2.3.2 Verification of autoantibody specificity by pre-absorption experiment

2.3.2.1 Purification of $(His)_6$-survivin protein

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 targeted protein is produced in fusion with His-tag sequence, it can be purified using a solid support that is covalently modified to display a heavy metal like Ni^{2+} on the surface. After producing $(His)_6$-survivin protein in a large-scale, bacterial cell lysate was prepared and purified with Ni^{2+}-charge His•Bind Resin under denaturing conditions using 6 M urea. Crude cell lysate was solubilized in 0.5X binding buffer and applied onto Ni^{2+}-charge resin, after which it is washed with binding buffer containing 5 Mm imidazole and washing buffer containing 20, 40, 60 and 80 Mm imidazole before being eluted with 500 Mm 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 21, the result showed that although wash buffer containing 20, 40, 60 and 80 Mm of imidazole can get rid a lot of non-specific proteins bound to Ni^{2+}-charge resin (lane 3-6, Figure 21), a little of proteins still remained contaminant in the elution fraction (lane E1, Figure 21).

During the purification step, fraction of flow-through was also collected and subjected to analysis, as shown in lane 1 (Figure 21). In this study, from 500 ml of bacterial culture, 50 ml of cell lysate was prepared and subjected to affinity chromatography. After the purification process, the obtained $(His)_6$-survivin was dialyzed against PBS, pH 7.4 at 4 ^{o}C overnight to get rid of imidazole.

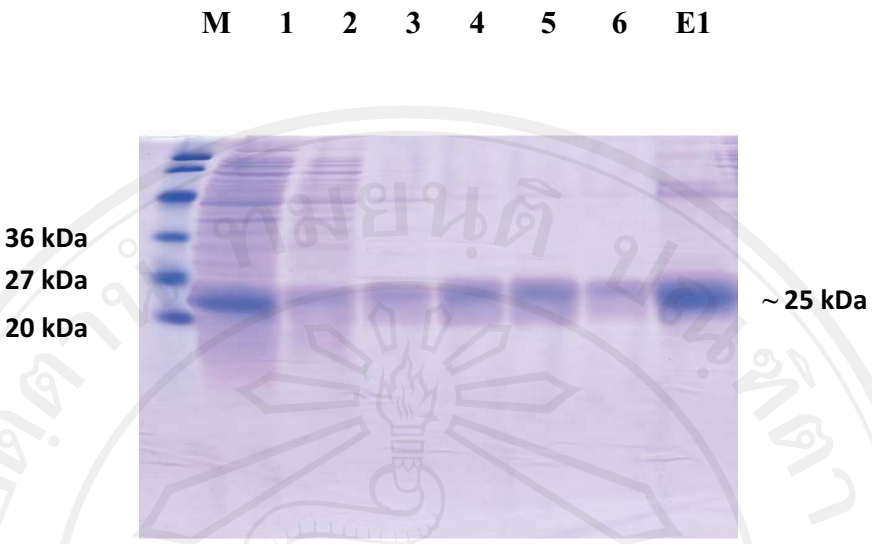

Figure 21. Coomassie blue stain of polyacrylamide gel showing preliminary purification process of His_6-survivin fusion protein using affinity chromatography. Fractions of flow-through were analyzed by SDS-PAGE, followed by Coomassie blue staining. The expected molecular mass of the purified protein was ~25 kDa.

- Lane M = protein molecular weight marker
- Lane 1 = fraction of crude cell lysate
- Lane 2 = fraction of flow through which was washed by binding buffer
- Lane 3 = fraction of flow through which was washed by washing buffer containing 20 Mm imidazole
- Lane 4 = fraction of flow through which was washed by washing buffer containing 40 Mm imidazole
- Lane 5 = fraction of flow through which was washed by washing buffer containing 60 Mm imidazole
- Lane 6 = fraction of flow through which was washed by washing buffer containing 80 Mm imidazole
- Lane E1 = fraction of eluted His_6-survivin protein

2.3.2.2 Pre-absorption of anti-survivin antibody with purified $(His)_6$-survivin protein

To determine the specificity of survivin ELISA, 3 positive sera were pre-absorbed with purified recombinant survivin protein at concentration 0, 25, 50 and 100 μg/ml after that which subjected to ELISA. Data were obtained in triplicate for each sample. It was found that the reactivity of anti-survivin mAb and test sera decreased after pre-absorption with high concentration of purified recombinant survivin protein. The results showed in Figure 22(A) and (B).

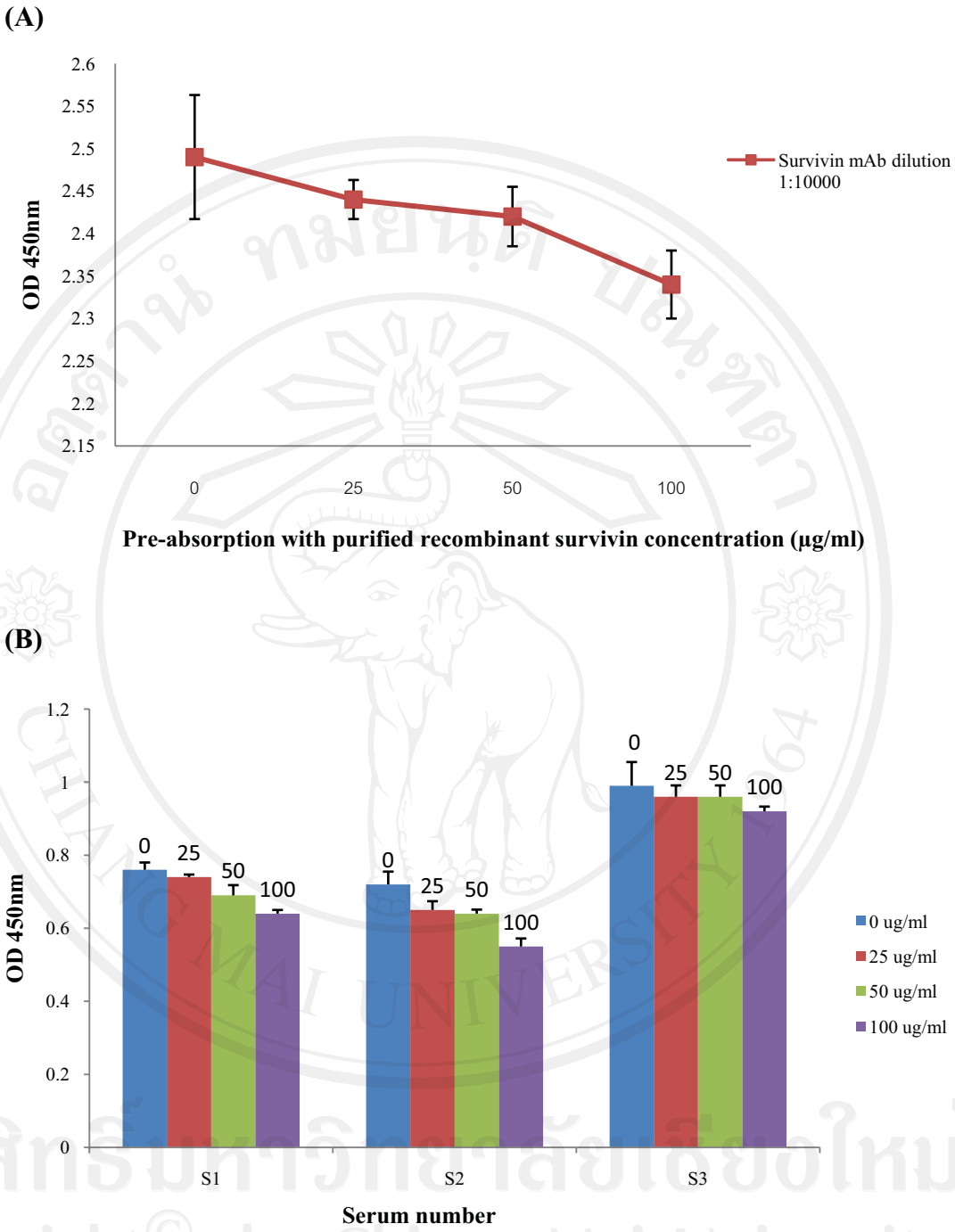

Figure 22. Preabsorption of anti-survivin mAb and sera from lung cancer patients with the purified recombinant survivin protein. Anti-survivin mAb at dilution 1:10000 (A) and three positive sera from lung cancer patients (S1, S2 and S3) at 1:1000 dilution (B) were tested in the survivin ELISA after preabsorption with various concentrations of purified recombinant survivin. Each bar or point represents the mean value of three determinations.

2.4 Detection of livin and survivin autoantibodies in lung cancer patient sera using the generated $(His)_6$-livin and $(His)_6$-survivin proteins

The reactivity of antibodies from lung cancer patient sera with the produced $(His)_6$-livin and $(His)_6$-survivin proteins were examined by using ELISA. Two hundred and fifty sera of lung cancer patients from Maharaj Nakorn Chiang Mai Hospital and fifty-five normal sera of healthy blood donors were analyzed.

The cut off value for anti-livin positivity was determined from healthy donor samples as the mean absorbance +2SD, which was 0.763. It was found that 27 of 250 lung cancer patients (10.8%) were positive (Figure 22A).

In addition, the cut off value for anti-survivin positivity was also determined from healthy donor samples as the mean absorbance +2SD, which was 0.593. Fourty-four sera of 250 (17.6%) were found to be positive (Figure 22B).

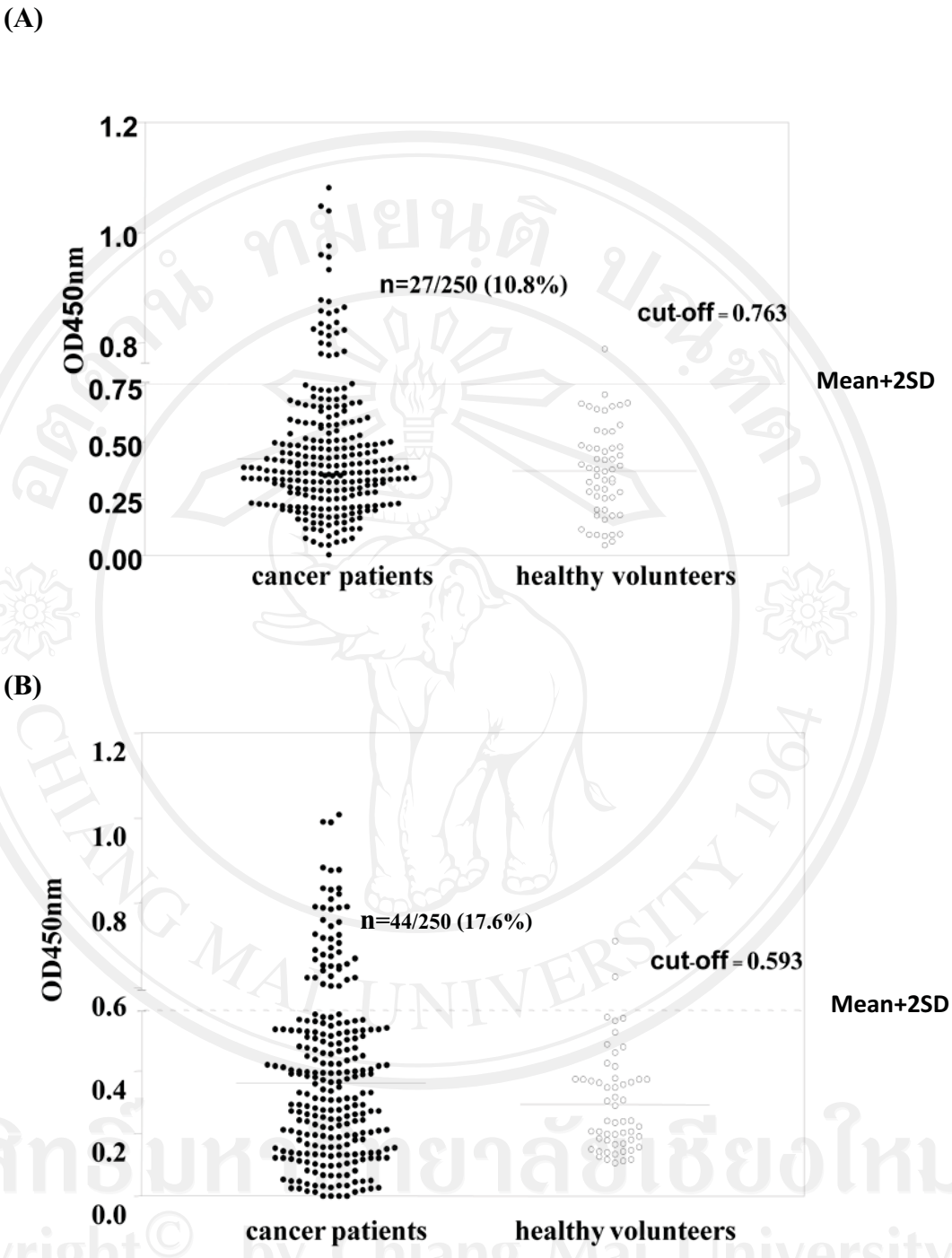

Figure 23. Detection of antibodies response to livin (A) and survivin (B) by established ELISA. Two hundred and fifty serum sample from lung cancer patients were analyzed for reactivity with immobilized $(His)_6$-livin and $(His)_6$-survivin recombinant proteins, respectively, using the optimized conditions as described in the text.

2.5 Correlations between anti-livin, anti-survivin antibodies positivity and clinicopathologic features

The presences of anti-livin and anti-survivin antibodies upon diagnosis were analyzed in correlation with clinicopathological parameter (sex, stage of disease, metastasis and smoking history). As shown in Table 2, no significant correlations were identified against any parameter examined, with the exception of a strong significant relation with anti-survivin positivity and an early stage (stage I) of disease (p=0.005).

Table 2 Correlations of anti-livin and anti-survivin antibodies levels upon diagnosis with clinicopathological parameters in all patients (total number of cases; n=42), ***** Chi-square test

Clinicopathological parameter	**Anti-livin antibody**		**Anti-survivin antibody**	
	n (positive/total)	***p*-Value ***	**n (positive/total)**	***p*-Value ***
Sex				
Female	4/19	0.488	2/19	0.802
Male	3/23		3/23	
Stage of disease				
I	3/13	0.458	5/13	0.005
II	1/6		0/6	
III	0/9		0/9	
IV	3/14		0/14	
Metastasis				
No	3/28	0.143	5/28	0.092
Yes	4/14		0/14	
Smoking history				
Yes	7/35	0.195	5/35	0.287
No	0/7		0/7	