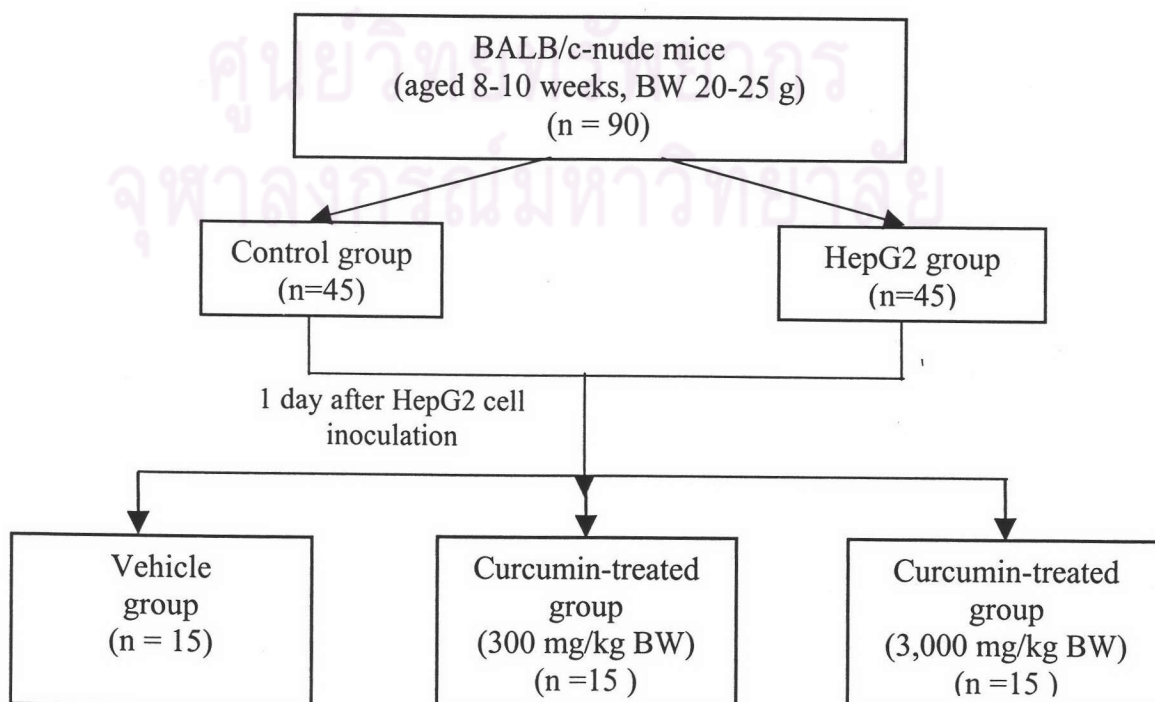# CHAPTER III

MATERIALS AND METHODS

Animal preparation

Male BALB/c-nude mice 8-10 weeks of age (National Laboratory Animal Center of Salaya Campus, Mahidol University, Thailand) weighing about 20-25 g were used. They were allowed to rest for a week after arrival at the Animal Center, Department of Physiology, Faculty of Medicine, Chulalongkorn University before being used in the experiment. The animals were kept in a pathogen limited room where temperature was 25 ± 3 °C. During the experiments the animals were housed one animal per cage with free access to sterilized water and standard laboratory chow, and with a 12 hours light and dark cycle. For ethical consideration, our experiments were performed following "Home office quidelines on the scientific use of animal (Scientific procedures) Act 1986" and approved by Ethical Committee, Faculty of Pharmaceutical Science, Chulalongkorn University.

All animals were divided randomly into two groups of HepG2 (HepG2) and control (Con). Then, they were further separated into 3 subgroups of vehicle, treated with 300 and 3,000 mg/kg BW of curcumin as follows:

```
                    BALB/c-nude mice
                (aged 8-10 weeks, BW 20-25 g)
                         (n = 90)

        Control group                    HepG2 group
          (n=45)                           (n=45)

              1 day after HepG2 cell
                    inoculation

    Vehicle          Curcumin-treated       Curcumin-treated
     group                group                  group
   (n = 15)         (300 mg/kg BW)         (3,000 mg/kg BW)
                       (n =15 )                (n =15 )
```

Dorsal skin fold chamber preparation

In this study, the dorsal skin-fold chamber nude mice model was used to study the growth and microvascularization of transplanted HepG2. The model of dorsal skin-fold chamber has been used for the intravital microscopic investigation of skin in nude mice over a minimum period for two weeks (Lehr *et al.*, 1993).

For the surgical procedure, the animals were anesthetized (sodium pentobarbital 50 mg/100 g BW, i.p.). Prior to chamber implantation, the entire back of animals was depilated, and two symmetrical stainless steel frames were implanted so as to sandwich the extended double layer of the skin. One layer of the skin fold was removed in a circular area of approximately 7 mm in diameter. The remaining layer, consisting of epidermis, subcutaneous tissue, and the striated skin muscle, was covered with a cover slip incorporated into one of the frames (Figure 3.1). All surgical procedures were performed under aseptic conditions.
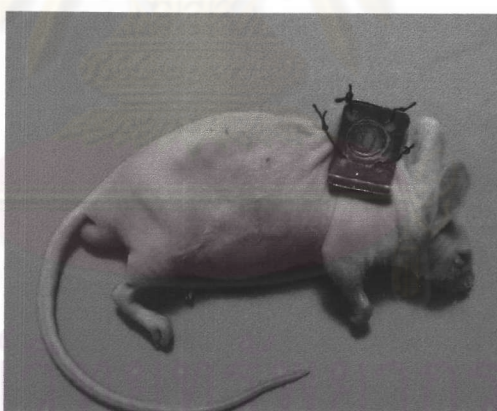

Figure 3.1. The animal with dorsal skin fold chamber

HepG2 human hepatocellular carcinoma cells, 30 µl of Hep G2 cells (2×10^6 cells, viability 95-97%) were inoculated into the middle area of the chamber hole as showed in the Figure 3.2.
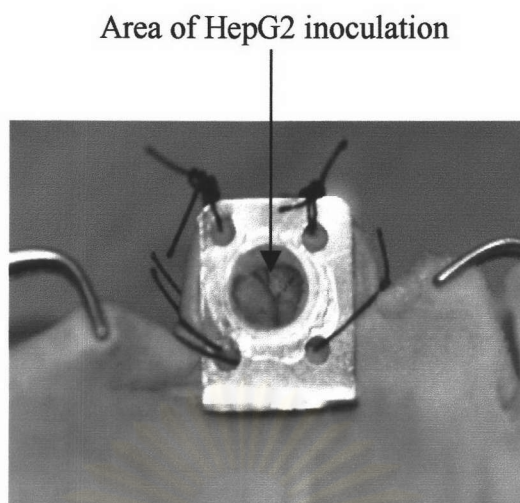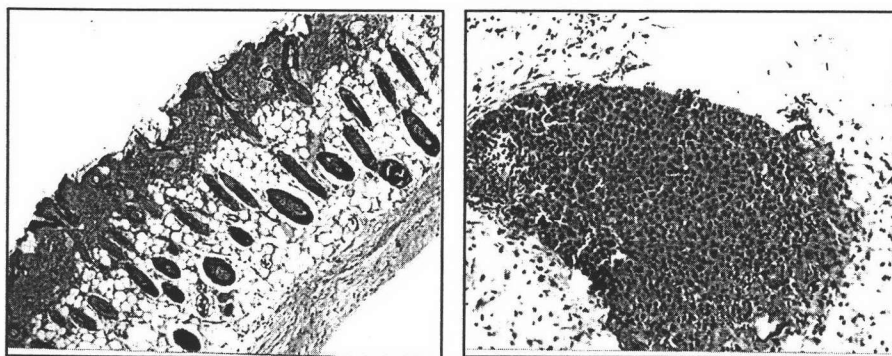
Area of HepG2 inoculation

Figure 3.2. The animal with dorsal skin fold chamber and area of HepG2 inoculation

The human hepatocellular carcinoma cell lines (HepG2) were originally obtained from the American Type Culture Collection (ATCC). Passage numbers for HepG2 was between 250 and 255 were used. Cell passages were prepared at the Research and Development Institute, the Government Pharmaceutical Organization, Bangkok, Thailand.

By histological examination, the result shown that HepG2 cells differentiated during 24 hours after the inoculation (Figure 3.3). Therefore, the treatments were started 24 hours after HepG2 inoculation. In Con-cur and HepG2-cur groups, the mice were daily oral treated by 20 ml of 300 or 3,000 mg/kgBW of curcumin (Sigma Chemical, USA) by gavage (Singh *et al.*, 1998; Huang *et al.*, 1994; Plummer *et al.*, 1999; Kawamori *et al.*, 1999) daily until the day of the experiment. In Con. and HepG2 groups, the mice received vehicle (0.1% DMSO). The concentration of DMSO (maximal 0.1% v/v) did not interfere with cell viability (Ireson *et al.*, 2001).

Control   1 day HepG2 inoculation

Figure 3.3. Microphotographs of an area of tissue in the dorsal skin-fold chamber (stained with hematoxylin-eosin) before (control) and one day after HepG2 inoculation. No remarkable skin and subcutaneous tissue appear, while the HepG2 section (right) shows positive deposit of malignant cells

Experimental Protocol

The experiments were performed days 3, 7, and 14 after vehicle or curcumin treatments. On the day of experiment, mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/ kg BW). A catheter was inserted into a jugular vein for fluorescence tracer application. Then the dorsal skin-fold chamber was removed and skin area around the chamber was fixed with modeling wax on a plate.

The tumor microvasculature was observed under an intravital fluorescence microscope (Nikon, Japan) equipped with a videocamera (Sony SIT68, Japan). An objective lens of 10x was used. 5 min prior to the observation, 0.1 ml of 0.5% rhodamine B isothiocyanate-labeled dextran (RITC-dextran) (Sigma Chemical, USA) was injected intravenously to visualize the intralumen of microvessels. The interested areas within the tumor-bearing chambers were recorded using a video-recorder (Sony SVT-124P, Japan).

The recorded videoimages were analyzed to calculated microvascular parameters (neocapillary density and mean capillary diameter), using a digital image processing software (Global LabII).
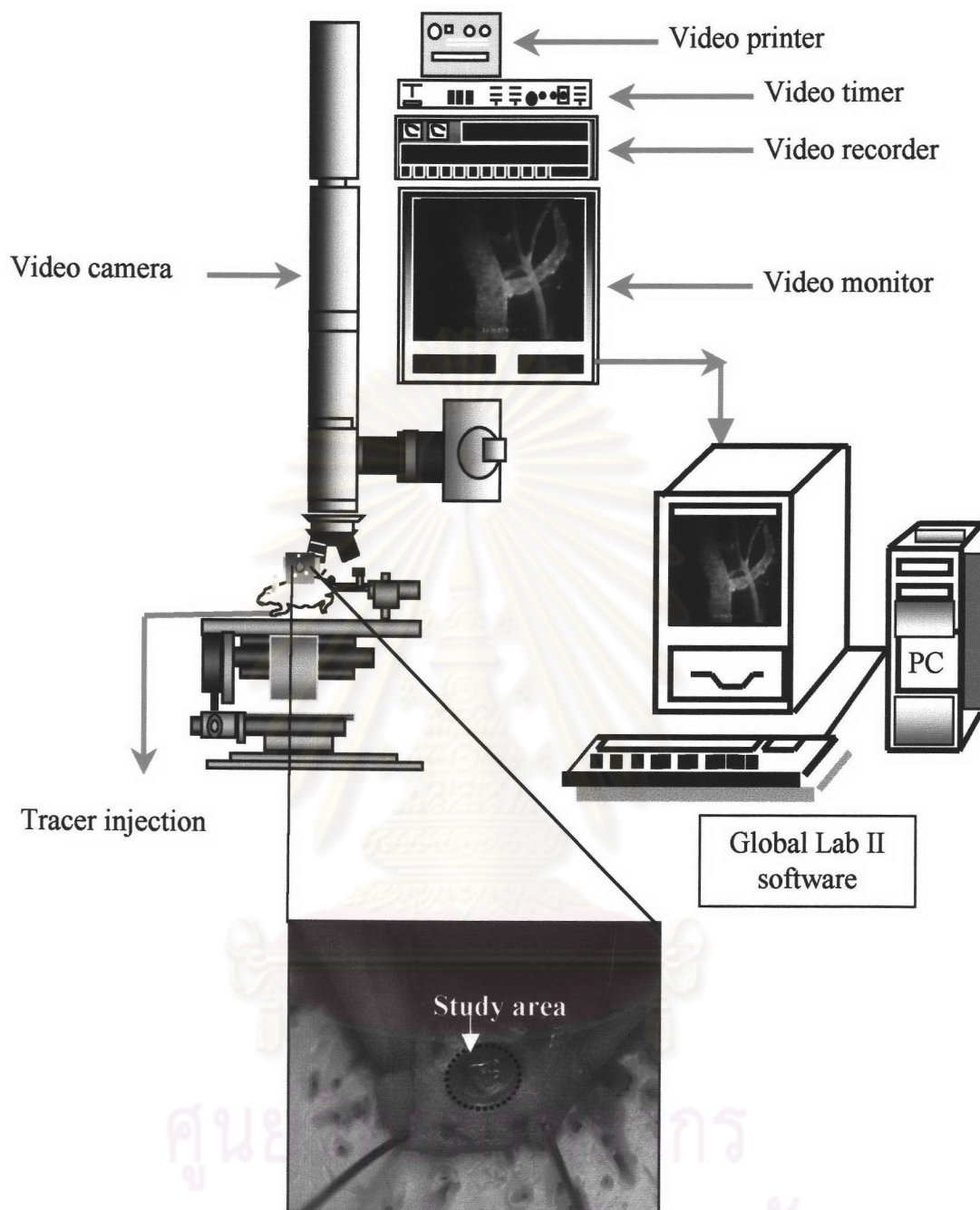
Figure 3.4. Schematic of a setup for intravital fluorescent microscopy of the dorsal skin fold chamber microvasculature in the mice.

Measurement of microvascular parameters

Neoapillary density (NCD)

1. From videoimages of each experiment, 10 videoframes containing clear capillaries network were randomly selected.

2. Each videoframe, 7-8 clear window frames approximately 10,000 pixel size (as shown in Figure 3.5A), was selected to determine capillary density. The window frame which covered only capillary network with aware the large vessels was selected to avoid the artificial result.

3. Each selected window frame, the minimum and maximum pixel within the capillary were determined. The numbers of pixels located within the capillaries were automatically calculated by using Global LabII software.

4. The ratio of the number of pixels within the capillaries to total number of pixels within the selected window (~ 10,000 pixels) was represented in the percentage, using the "Histogram" functional tool as determined in Figure 3.5 B.
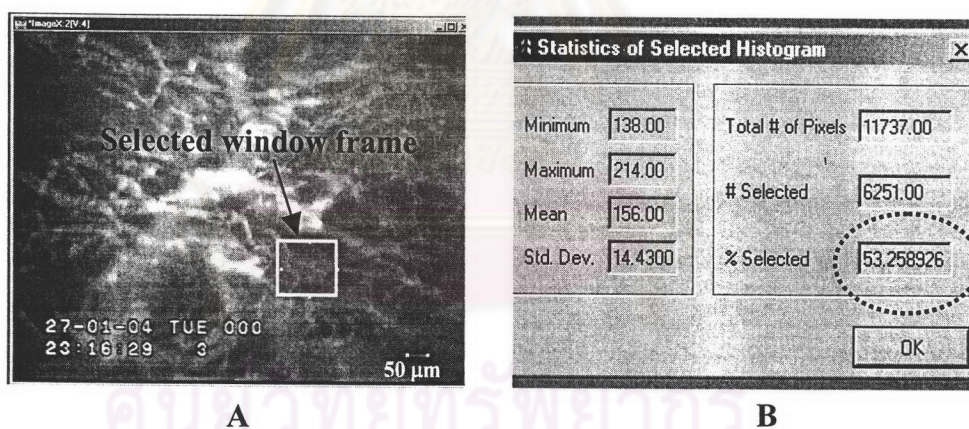

A   B

Figure 3.5. An example of videoimage containing clear neocapillaries network, which was selected randomly from videoimages of each experiment (A). The "Histogram" functional tool, which used to calculated the percentage of neocapillary density (B).

Using the above procedures, we defined the NCD as follows:

$$NCD = \frac{(Number\ of\ pixels\ within\ the\ capillaries)\ x\ 100\ (\%)}{(Total\ number\ of\ pixels\ within\ the\ selected\ area)}$$ **Equation 1**

Based on the recorded videoimages, the CD was evaluated, and expressed in the percentage. Fro each mouse, the CD was measured at 70-80 window frames to provide the average level at different periods after the inoculation of tumor cells. The CD level was used as an index of angiogenesis.

Capillary diameter

Capillary diameter was measured with a Global Lab device. After stabilization, a single capillary with the diameter of 5-15 μm were chosen for observation. By using the reference point, the diameter measurement was able to confine within the same location (Figure 3.6). The neocapillary diameter in micrometer (μm) was calculated as the mean of triple measurements from three video frames.
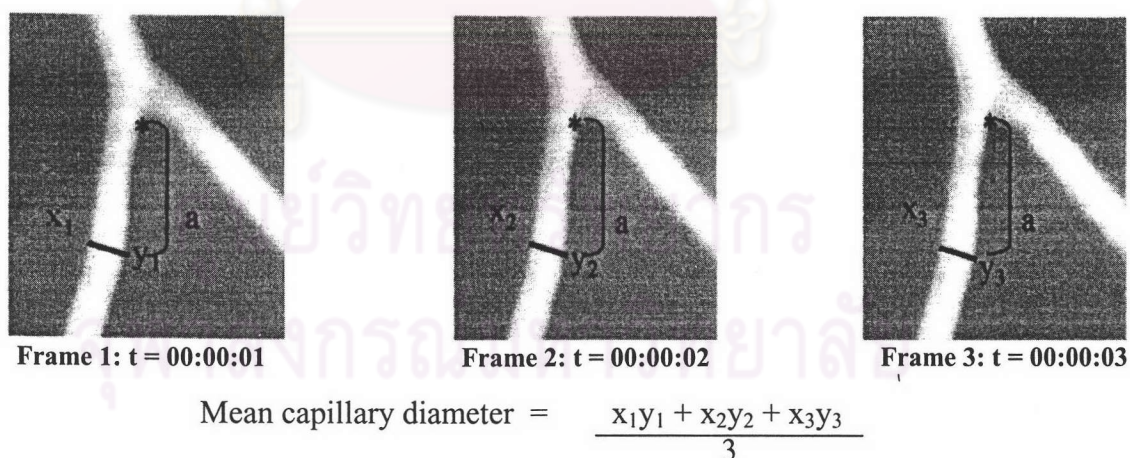

| Frame 1: t = 00:00:01 | Frame 2: t = 00:00:02 | Frame 3: t = 00:00:03 |

$$Mean\ capillary\ diameter = \frac{x_1y_1 + x_2y_2 + x_3y_3}{3}$$

Figure 3.6. Method for measurement of capillary diameter

Tissue blood perfusion

Blood flow perfusion of an area of tissue within the dorsal skin-fold chamber (Figure 3.7A) was measured using a Doppler flowmetry (Model ALF21, USA)(Figure 3.7B) with a probe (1mm diameter)(Figure 3.7C). The measured value was expressed in arbitary unit (AU). For each mouse, the blood perfusion was measured at 10 points (avoid large vessels) within the dorsal skin-fold chamber to provide the average level at different period after the inoculation of tumor cells.
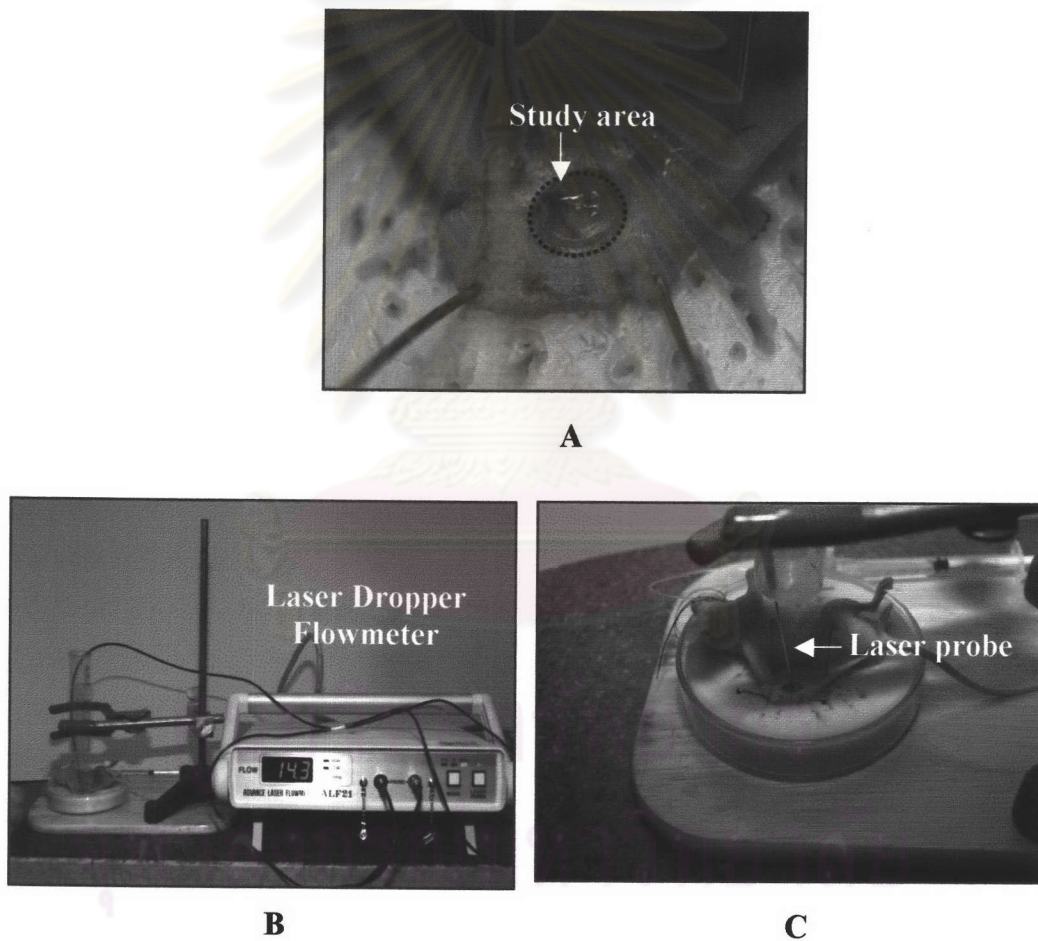

A

B   **C**

Figure 3.7. Measurement of tissue perfusion by using a Doppler flowmetry of an area of tissue within the dorsal skin-fold chamber

Enzyme Immunoassay (EIA) for Serum VEGF

Blood sample obtained from the cardiac puncture was collected in a microcentrifuge tube and allowed to clot for half an hour, after which it was centrifuged at 5,000 rpm for 10 minutes. The serum was separated and stored at -20^0C until analysis for VEGF was performed. This assay uses the *ChemiKine's* assay system. *ChemiKine*^{TM} Mouse VEGF kit (Chemicon International, Inc., CA) is a "competitive" enzyme immunoassay (EIA), which measures the total amount of the VEGF in serum. With the *ChemiKine*^{TM} assay system, precoated goat anti-rabbit antibody plates are used to capture a specific VEGF complex in each sample consisting of rabbit anti-mouse VEGF antibody, standard VEGF or unknown, and biotinylated VEGF. The biotinylated VEGF conjugate (competitive ligand), and or VEGF in the sample compete for VEGF specific antibody binding sites. Therefore, as the concentration of VEGF in the sample increases, the amount of biotinylated VEGF captured by the antibody decreases.

The assay is visualized using a streptavidin alkaline phosphatase conjugate and an ensuing chromagenic substrate reaction. This assay uses a two-step color generating system. In this system, alkaline phosphatase dephosphorylates NADPH (substrate) to NADH. The NADH then serves as the cofactor, which activates a cycling redox reaction driven by alcohol dehydrogenase and diaphorase. The latter reaction forms a deep red colored product (formazan), which absorbs light at 490 nm. The amount of VEGF detected in each sample is compared to a VEGF standard curve which demonstrates an inverse relationship between Optical Density (O.D.) and cytokine concentration: i.e. the higher the O.D. the lower the cytokine concentration in the sample. Each standard and sample was assayed in duplicate. The minimal detectable amount of serum VEGF by this kit is 0.49 ng/ml, and the maximum undiluted amount detected is 500 ng/ml.

The condition of serum VEGF analysis, a standard curve was generated by 6 serial concentrations of 0.49, 1.95, 7.81, 31.25, 125, 500 ng/ml from Lyophilized Standard. The following protocol recommended by manufacturer was used to make up the kit standard in the serial dilutions.

1. Label 6 12x75 test tubes #2-6 and 0 dose (#7). Add 750 µl of the diluent to the 6 Standard tubes.

2. Reconstitute the VEGF Standard with 2,000 µl of the diluent and vortex. This is Standard #1, which has a concentration of 500 ng/ml.

3. Standard #2-6 are then prepared by performing a 1:4 dilution of the preceding standard. For example, to make Standard #2, adding 250 µl of Standard #1 to tube #2, vortex then continue transferring 250 µl to next tube. Do not add any VEGF Standard to the "0 dose" Standard tube (#7).
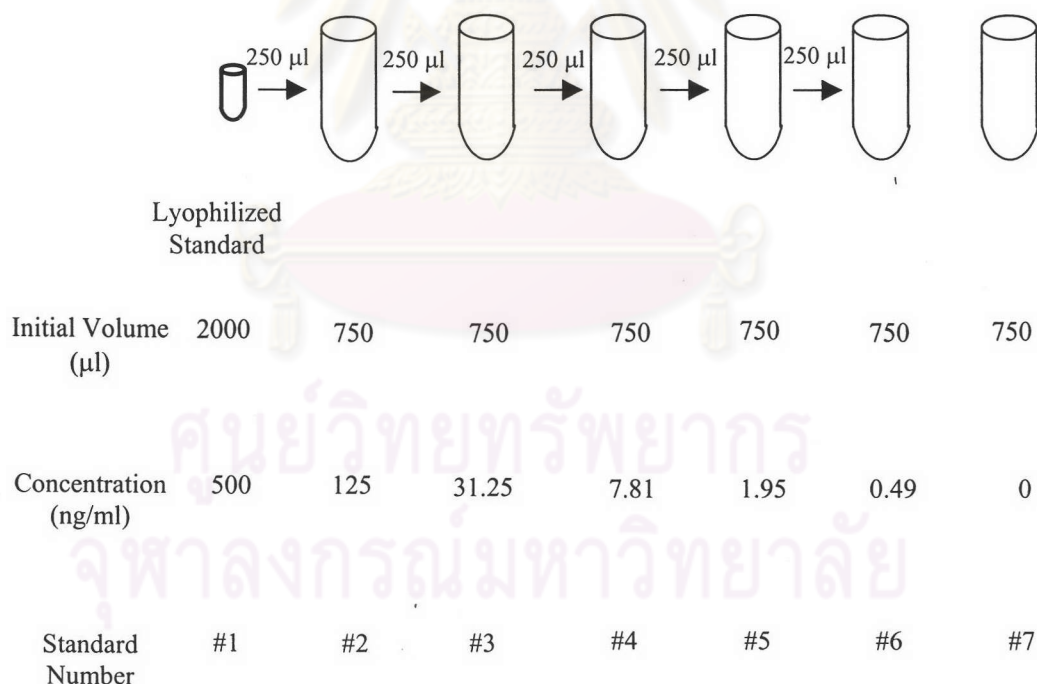

	Lyophilized Standard						
Initial Volume (µl)	2000	750	750	750	750	750	750
Concentration (ng/ml)	500	125	31.25	7.81	1.95	0.49	0
Standard Number	#1	#2	#3	#4	#5	#6	#7

Figure 3.8. Serial dilution of VEGF Standard.

VEGF Assay

The experimental protocol as performed by the sequential step as following:-

1. In duplicate, dispense 100 µl of the standard #1-7 into their designated wells.

2. For each individual sample, add 100 µl of sample + 100 µl of diluent (1:2 dilution). In duplicate, to each of the designated well, dispense 100 µl of each diluted serum sample preparation.

3. Dispense 25 µl of reconstituted mouse VEGF Conjugate into each well. Proceed immediately to next step.

4. Dispense 25 µl of reconstituted rabbit anti-mouse VEGF polyclonal antibody into each well. Seal plate with acetate plate sealer to prevent evaporation and incubate for 3 hours at room temperature.

5. Gently remove the plate sealer and wash the plate 5 times. A thorough washing of the plate is extremely important to reduce background. Using a multi-channel pipette to fill each well with 250 µl of dilute wash buffer, flick and blot the plate. Repeat this procedure for a total of 4 times. Dispense 250 µl of dilute wash buffer a fifth time and let plate soak for 10 minutes. After that, blot and aspirate each well to remove any excess fluid.

6. Dispense 50 µl of the dilute streptavidin-alkaline phosphatase into each well. Reseal the plate and incubate at room temperature for 30 minutes.

7. Gently remove the plate seal. Wash the plate 5 times similar to describe above in 5.

8. Dispense 200 µl of the color reagent solution into each well. Incubate for 20 minutes at room temperature.

9. Read the plate at 490 nm, when the Optical Density (O.D.) for "0 dose" reached 1.6. Dispense 50 µl of stop solution at this

time into each well, then O.D. was determined throughout the plate.

The serial VEGF standard concentrations and the corresponding O.D.'s were demonstrated in Table 3.1.

Standard VEGF Concentration (ng/ml)(x)	0.49	1.95	7.81	31.25	125	500
log (x)	-0.310	0.290	0.893	1.495	2.097	2.699
OD	1.867	1.6375	1.5125	0.9435	0.73	0.4245

Table 3.1. The optical density of serial VEGF standard concentrations

Calculation of Results

To make the VEGF standard curve, known concentrations of VEGF are plot on the log scale (X-axis) and the corresponding O.D. on the linear scale (Y-axis). The standard curve shows an inverse relationship between VEGF concentrations and the corresponding O.D.'s (absorbance). In other words, the greater the concentration of VEGF in the sample is, the lower O.D., or less red color is observed. The concentration of VEGF in unknown samples was evaluated from the standard eqation of log (x) = 3.508-1.952(y) as shown in Figure 3.9.
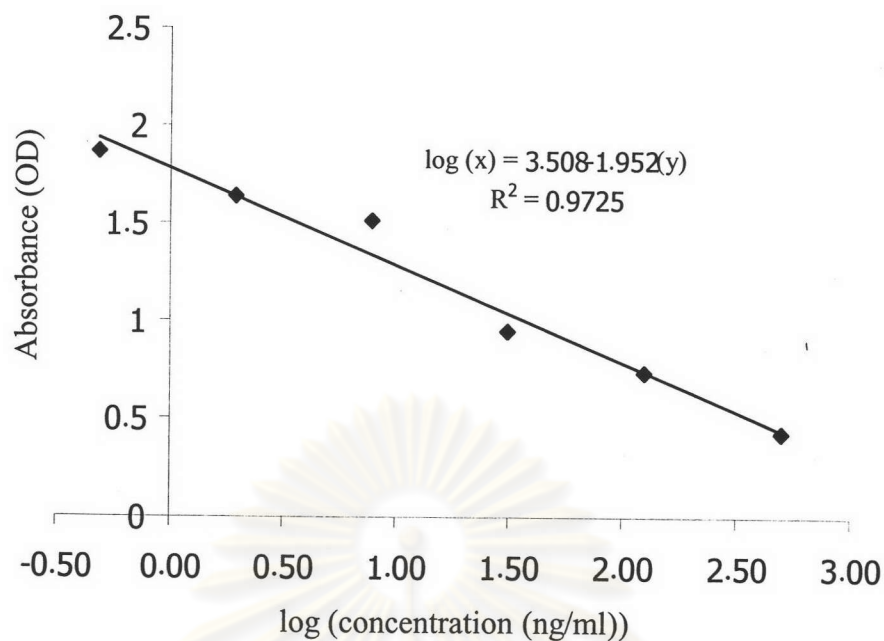
Figure 3.9. The standard curve for VEGF level

Determination of COX-2 Protein by using Western Blot Analysis

Protein Extraction

Tissues samples within the dorsal skin-fold chamber were excised and fragmented with medium-sized scissor on ice block, containing homogenization buffer (50 mM Tris buffer, 10 mM EDTA, 1% Triton X-100 with 10 mM Phenylmethylsulfonyl fluoride, 4 μg/ml Pepstatin and 0.5 μg/ml Leupeptin). Then, tissue fragments were homogenized in an ice bath using homogenizer rotated at 13,000 rpm for 3 minutes.

Homogenates were centrifuged at 3,500 rpm 4°C for 40 minutes to remove tissue debris. The supernatant was collected and aliquot into sterile microcentrifuge tubes, kept at -80°C until used. All samples will be further assayed for the amount of protein and Western blot analysis.

Protein Assay (Lowry Method)

Principle

The protein content was determined by using Lowry method (Lowry *et al.*, 1951). The Lowry method for determining protein concentration is essentially a biuret reaction that incorporates the use of Folin-Ciocalteu reagent for enhanced color development. The Lowry procedure is more commonly used in research applications, 100% correlation to the original Lowry assay, because it is ten times more sensitive than the biuret reaction. In the Lowry method, protein is first treated with alkaline copper sulfate in the presence of tartrate. This "incubation" is then followed by addition of the Folin-phenol reagent. It is believed that the enhancement of the color reaction in the Lowry procedure occurs when the tetradentate copper complexes transfer electrons to the phospho-molybdic/phosphotungstic acid complex (Mo^{+6}/W^{+6}, Folin phenol reagent). Reduction of the Folin phenol reagent yields a blue color read at 750 nm.

Chemical substances and reagents

1. Solution A (alkaline tartrate reagent)

$NaCO_3$	10.0	g
$Na_2C_4H_4O_6.2H_2O$	0.1	g
NaOH	1.2	g
DW	500	ml

2. Solution B (0.5% copper sulfate)

$CuSO_4.5H_2O$	0.5	g
DW	100	ml

3. Solution C

    Solution A : Solution B = 50 : 1

4. Solution D (1 N folin phenol reagent)

    2 N folin ciocalteu phenol reagent : DW = 1 : 1

5. Standard protein solution

    BSA 1 mg/ml

    Range 10-100 µg/ml

Procedure

Five standard dilutions in duplicate (10, 25, 50, 75, and 100 μg/ml) were prepared by dissolving the stock standard BSA solution (1 mg/ml) as indicated in Table 3.2.

Tube	BSA (μl)	DW (μl)	BSA (μg/ml)
1	0	500	0
2	50	450	10
3	125	375	25
4	250	250	50
5	375	125	75
6	500	0	100

Note: DW = Double distilled deionized water

Table 3.2. Standard curve preparation

Five standard dilutions in duplicate (10, 25, 50, 75, 100 μg/ml) were prepared by dissolving the stock standard BSA solution (1 mg/ml) as indicated in Table 3. 0.5 ml of each standard dilution, sample dilution and blank tubes (DW) were added into the clean 13 x 100 mm test tubes. Then 5 ml of solution C in each tube was added and mixed by vortexing and incubated at room temperature for at least 30 minutes. After that, quickly added 0.5 ml of solution D to each tube, vortexed immediately, and allowed to stand 30 minutes at room temperature. The absorbance of each solution was read and recorded at 750 nm against a reagent blank by using 1 ml cuvettes. The linear regression equation of a standard curve was used to calculate the concentration of protein in each sample.

Western Blot Analysis

Immuno-blotting is a widely used and powerful technique for the detection and identification of protein using antibodies. The process involves the separation of sample proteins by polyacrylamide gel electrophoresis (PAGE) followed by the

transfer of the separated proteins from the gel onto a thin support membrane. The membrane binds and immobilizes the proteins in the same pattern as in the original gel. The membrane (or "blot") is then exposed to a solution containing antibodies that recognize and bind to the specific protein of interest. The antibodies bound to the membrane are detected by any of a variety of techniques, usually involving treatment with a secondary antibody.

1. Separation of Protein (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis : SDS-PAGE)

The most widely used method for qualitative analysis of a protein mixture is SDS-PAGE using the buffer system of Laemmli (1970). With this method, it is possible to determine both the purity and the relative molecular weight of an unknown isolated protein or proteins. In the process, proteins migrate in response to an electrical field through pores in the gel matrix and separate based on molecular size after sample proteins are solubilized by boiling at $100^{o}C$, 5 minutes in the presence of anionic detergent, SDS detergent and 2-Mercaptoethanol (2-ME). The 2-ME is a disulfide reducing agent, and serves to reduce disulfides holding together the tertiary structure of the protein. The anionic SDS detergent binds strongly to the protein thus disrupting its secondary, tertiary and quaternary structure, resulting in a linear polypeptide chain coated with negatively charged SDS molecules. The binding efficiency of the SDS is generally one SDS molecule for every two amino acid residues. Under this condition, the polypeptide chains are unfold and assumed a rod-like structure and have negative charge, resulting in a constant charge to mass ratio. Then proteins move through a polyacrylamide gel matrix toward the anode. The polyacrylamide gel is cast as a separating gel topped by a stacking gel and secured in an electrophoresis apparatus. Separation is determined by size and therefore when compared to standards of known molecular weight, the relative molecular mass can be estimated. Purity is determined by the presence of a band associated with the desired protein and the absence of bands associated with contaminating proteins.

The SDS gel is comprised of a main separating gel and a stacking gel. The proteins, which have been mixed with a loading buffer, containing an ionizable tracking dye bromophenol blue, are loaded into wells formed in the stacking gel. A current is passed through the gels and the proteins migrate through the stacking gel

and are concentrated into a solid band at the separating gel. When the proteins enter into the separating gel, the negatively charged protein-SDS complexes migrate toward the anode. Their migration in the gel relative to each other is the same based on their uniform negative charge. Separation, therefore, occurs as a result of the molecular sieving properties of the gel. The larger the protein, the more its mobility is retarded by the frictional resistance of the gel the smaller the molecule the further its mobility in the gel. The bromophenol blue is completely unretarded in the gel due to its small size relative to proteins and it is thus used to monitor the progress of the electrophoresis. The current is turned off once the tracking dye has migrated to bottom of the gel. The gel is removed from between the plates and the stacking gel discarded. The separating gel is stained by shaking it in a solution of Coomassie Brilliant Blue for a few hours and then destained by shaking overnight to remove the unbound dye background. The proteins are visible as blue bands on a clear background.

1.1 Assembly of Apparatus

The reagents used in preparing the gel should be removed from the refrigerator and allowed to warm and degas for one hour prior to the preparation of the gel. While this is happening, set up the "sandwish" plates for casting the gel itself. A "sandwish" consists of one outer is a rectangular back plate with the rounded bottom corners, measuring 10 x 11.5 cm glass plate and one inner is the same size but with a notch 1.1 cm deep and 10.4 cm long separated by plastic spacers of equal thickness (0.5 mm).

1.2 Preparing of Slab Gel

The polyacrylamide gel was prepared using N,N-methylenebis acrylamide as cross-linker in the amount corresponding to 2.6% of the weight of acrylamide. The separating gel of 7.5 % acrylamide (see Appendix A) was carefully poured between the plates so that it flows down the slide between plates and fills from bottom to top with no air bubbles. The comb adjusted the height of the separating gel. That height of the gel should be approximately 1 cm below the bottom of the comb. The polymerize was allowed to incubate at room temperature for at least one hour.

After the separating gel is solid (polymerize) then the 5% stacking acrylamide (see Appendix A) gel would be prepared. The water was drained off and the excess liquid was removed with a piece of Whatman 3 MM paper. The correct comb (number of wells, thickness same as spacers) with ten teeth was immediate gently inserted between the surfaces of lower gel to create lanes for adding sample. It should be made sure that no air bubbles form around the teeth of the comb, as they will impede the migration and separation of the proteins. Then leaving the gel for one hour at room temperature prior to electrophoresis was performed.

1.3 Preparation of Sample

The samples (10 μg) were prepared to a final volume of 15 μl by adding sample buffer (see Appendix A) containing a tracking dye (bromophenol blue). Heated the samples for five minutes to $100^{\circ}C$ in a boiling water bath were performed to denature protein. The treated protein solution could be kept at $4^{\circ}C$ until loaded on gel. Unused sample can be stored at $-20^{\circ}C$ and boiled again before using. The markers do not need to be boiled as recommended by the Amershem pharmacia biotech company, UK.

2. Electrophoresis

After polymerization is complete, the samples (10μg/lane) were load up into polyacrylamide gel as described by Laemmli (1970). 10 μl of COX-2 protein standards (0.125 and 0.0625 μg of positive control, Cayman Chemical, USA) was added to separate lanes. The electrophoresis apparatus was attached to an electric power supply. The power supply was adjusted at 150 V. The gel was run until the dye front reaches the bottom of the gel, approximately one hour. Then the power supply was turned off. To make the orientation of the gel, a corner from the bottom of the gel was cut. After that the gel from the glass plate was transferred to a staining dish or buffer for transfer protein to membrane.

3. Staining and Destaining

Polypeptides separated by SDS-PAGE can be simultaneously fixed with methanol : glacial acid and stained with Coomassie Brilliant Blue (R-250, Sigma) (see Appendix A). The gel was covered with Coomassie staining solution and placed on the shaker and allows the gel to stain for at least two hours. Decant off the dye and add destaining solution to cover the gel was performed. Excess background stain was removed by soaking the gel in several changes of destaining solution (see Appendix A) until the background color was clear. This may take several hours. The proteins will appear as dark blue bands on a colorless background.

4. Drying

When destaining was complete, the gel was washed briefly in deionized water, transferred onto a cellophan sheet, which is arranged on glass plate and another piece of sheet on surface of the gel. The piece of cellophan sheet should be large enough to accommodate all of the gels that are to be dried at the same time. Then seal around the gel with clamps. Gently smooth out any air bubbles between sheets, which will interfere when drying. Leave the gel at room temperature for overnight.

5. Molecular Weight Determination

The molecular weight of an unknown protein was estimated by comparing its mobilities of known standards run on the same gel. A standard curve was constructed by plotting the relative mobilities of standard protein markers versus their log molecular weights on semilogarithmic graph paper.

Relative mobility of the polypeptides was calculated according to the following formula,

$$\text{Relative mobility} = \frac{\text{Distance of protein migration}}{\text{Distance of dye migration}}$$

The standard protein marker used in this study are the High Molecular Weight Calibration Kit for SDS electrophoresis composing of Myosin, 220 kDa ; α_2-

Macroglobulin, 170 kDa ; β-galactosidase, 116 kDa ; Transferin, 76 kDa ;and Glutamic dehydrogenase, 53 kDa. The standard curve is illustrated in Figure 3.10.
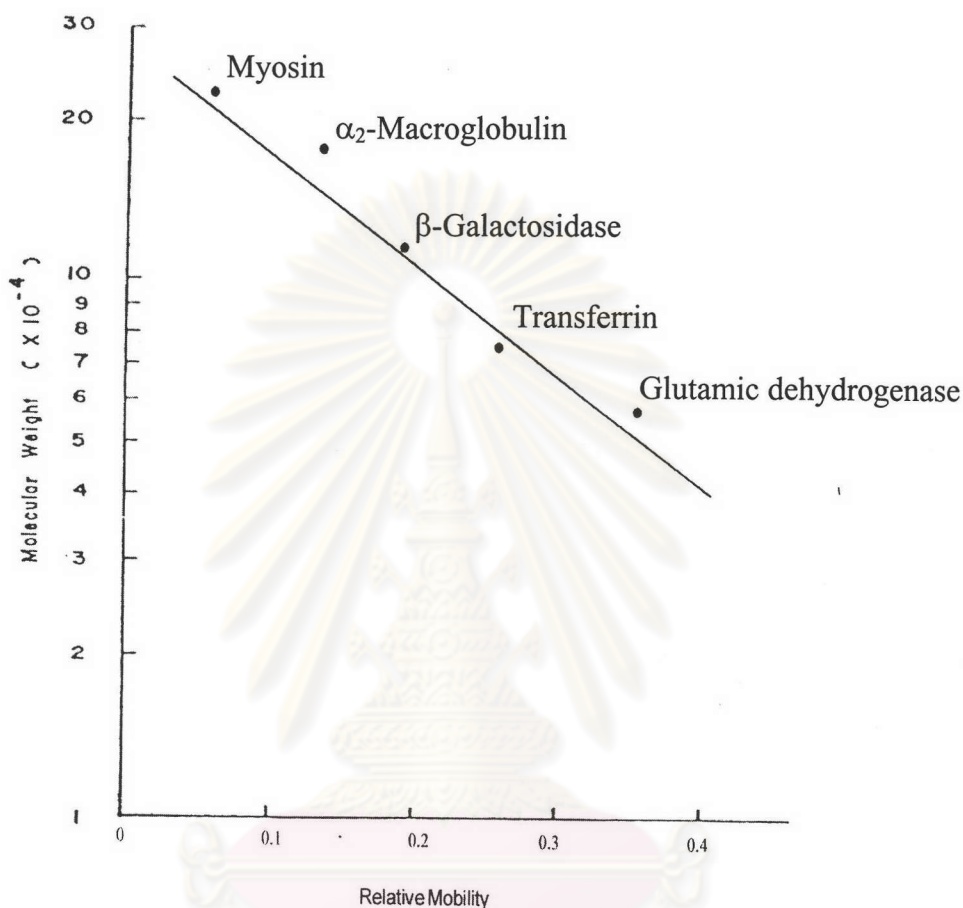

Figure 3.10. A calibration curve for molecular weight estimation by SDS-PAGE

6. Immunoblotting Technique

In this study, immunoblotting was used to detect the separated protein in SDS-PAGE. Western blot analysis is the most commonly used immunochemical technique, which is Towbin, in 1979, developed for studying protein function and localization. Unlike the blotting techniques of Southern and Northern, which are transfers of nucleotides.

In a Western blot, proteins are electrophoretically separated on an acrylamide gel, and then transferred to a membrane detected with one or more antibodies. The

antibody detection technique may be direct (an enzyme-conjugated tag-specific antibody) or indirect (first with an unconjugated tag-specific antibody, and with an enzyme-conjugated antibody). Then suitable enzyme substrates must produce a signal on the membrane at the site of enzyme-conjugated antibody by chemiluminescent (signal recorded on X-ray film) or chromogenic visualization (signal recorded on the membrane). In this study, COX-2 antigen extract was used as an antigen source.

6.1 Transfer Protein

After electrophoresis, an SDS-PAGE gel was transferred into transfer buffer (see Appendix A). Cut one sheet of Polyvinylidene difluoride (PVDF) membrane and two sheet of Whatman 3 MM paper to the size of the gel to be transferred, cut off the lower right corner of PVDF membrane to identify the gel and membrane orientation, and must be prewetted in absolute methanol for a few seconds, rinsed in DW. Sponge unit for electroblotting, Whatman 3 MM paper, and gel were immersed in a box containing transfer buffer. Assemble a sandwich of the components in the following order : 3 MM paper, SDS-PAGE gel, PVDF membrane, 3 MM paper, and sponge. Be careful to exclude any air bubbles between layers, which will interfere. The sandwich was placed between support pads provided with the transfer apparatus and inserted into the transfer device so that the membrane is closest to the side of the positive electrode. The transfer was done one hour at 100 volts. Then a current passed at right angles to the gel, which causes the separated proteins to electrophoresis out of the gel and into the PVDF membrane. This membrane is called "blot". After transfer, the membrane is removed from the sandwich and rinsed briefly in TBS (see Appendix A). The membrane can be stored dry, can be stained with Amido black staining solution (see Appendix A) to visualize proteins transferred, or used directly in the next step (blocking).

6.2 Blocking

The sensitivity of Western blotting depends on reducing background of nonspecific binding by blocking potential binding site with irrelevant protein. Place the PVDF membrane in a container, and add blocking solution (see Appendix A) incubated for two hours at room temperature with gentle agitation on a platform

shaker. After blocking, the membrane was washed 1 time 15 minutes, the 4 times 10 minutes with 80 ml of TTBS (see Appendix B).

6.3 COX-2 Antibody Detection

The dilution of the primary antibody was prepared in TTBS with 0.5% BSA. For COX-2 detection, Rabbit anti-COX-2 (murine) Polyclonal Antibody (Cayman Chemical, USA) at 1:1000 was used. Then the membrane was placed in a heat-sealable plastic bag. It was allowed to add 10 ml of primary antibody solution and incubated overnight at $4^{\circ}C$. After incubation the plastic bag was cut and the primary antibody solution was discarded. Washing the membrane were performed with 80 ml of TTBS in the period of 15 minutes once and 10 minutes for 4 times. The second antibody was prepared by diluting in TTBS with 0.5% BAS. For detection COX-2, Goat Anti-Rabbit IgG horseradish peroxidase (HRP) conjugate (Cayman Chemical, USA) at 1:10,000 was used. The membrane from the final wash in TTBS was transferred to a heat-sealable plastic bag containing 10 ml of fresh secondary antibody solution. Then the membrane was incubated for two hours at room temperature with gentle agitation on shaker. After that the bag was cut, the bag and the membrane was moved to a container, washed in 80 ml of TTBS once 15 minutes, 4 times 10 minutes, and 1 time 60 minutes with gentle agitation on a shaker.

6.4 Enhanced Chemiluminescence (ECL) Detection

The ECL detection system is a light emitting non-radioactive method for detection of immobilized antigens, conjugated directly with HRP-labeled antibodies. The chemiluminescent reaction of cyclic diacylhydrazides such as luminal has been widely used in chemical analysis and extensively studied. HRP is often used to catalyze the oxidation of luminal in the presence of hydrogen peroxide (H_2O_2). Immediately following the oxidation, the luminal is in an excited state, which may decay to the ground state via a light-emitting pathway.

ECL solution (see Appendix A) by combining solution A and solution B sufficient to cover membrane (use 5 ml of each solution per membrane) was prepared. In dark room, drain excess buffer from washed membrane and place membrane in a fresh container. Add the detection reagent directly to the membrane

on the surface carrying the protein; do not leave the membrane to dry out. Incubate for a few seconds at room temperature. The excess detection reagent was drained off and the membrane was gently placed, protein side up, on a piece of Saran Wrap. A piece of Saran Wrap was placed over the membrane, smoothing out any creases of bubbles that may develop between membrane and Saran Wrap. It is necessary to work quickly once the membrane has been exposed to the detection solution. The membrane was placed, protein side up, X-ray film cassette. The lights were turned off and a sheet of autoradiography film 18 x 24 cm (Hyperfilm™-ECL, Amersham Pharmacia Biotech, UK) was carefully placed on the top of the membrane, the cassette was closed and exposed for few seconds; this depends on the amount of target protein on the membrane. If background is high the membrane may be re-washed with TTBS and re-detected with ECL solution.

7. Image Analysis

For data analysis, the intensity of each band corresponding to COX-2 was analyzed by quantitative image analysis using Global Lab Image/2 software (see Appendix II).

Global Lab Image/2 (GLI/2) is an object-oriented scientific imaging software product that powerful, expandable, able to definite of contrasting areas, and improved precision in measurements, using the Edge Finder and Histogram tool to quantitate COX-2 bands. Results can be printed, exported to Excel spreadsheets and expressed in percent compared with control (100   ).

7.1 Image Requirements

The followings are the recommended requirements for using GLI/2 program : scanJet 6200C scanner (Hewlett Packard), CD writetable, picture 1200 dpi.BMP or JPEG files.

7.2 Step for quantitative COX-2 band

In this section, an example shows how to quantify COX-2 band by using GLI/2 program.

1. Begin by starting new View port (if its not open). From the file menu select New view port or from the Toolbar, select ☐ icon.
2. Next step will be opened a File Manager Tool. In the following example is the image file of Western blotting of COX-2 standard (Figure 3.11).
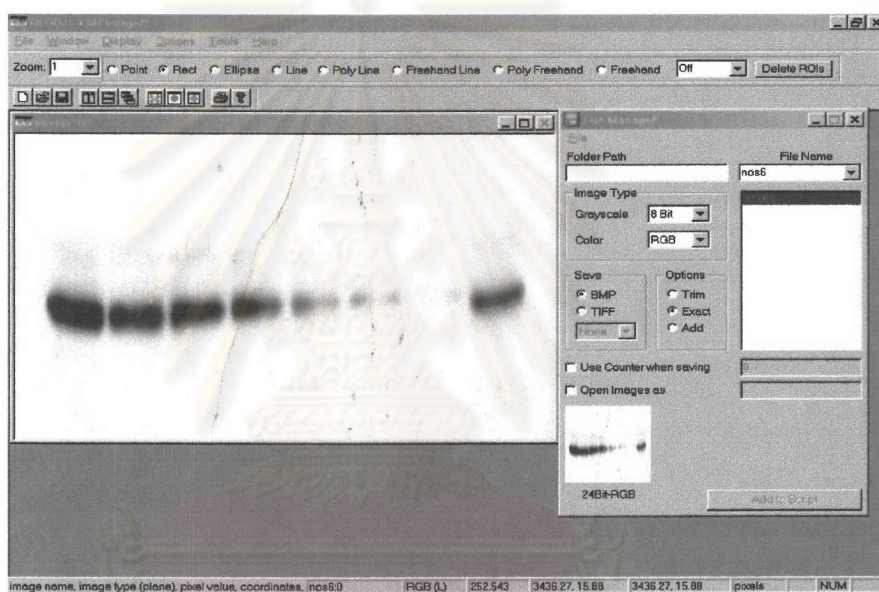

Figure 3.11. The image file of Western blotting outcome of COX-2 standard was analyzed by Global Lab II software.

3. Create a rectangle that covers around all COX-2 band by Rect in the ROI Menu bar and click Draw in the ROI actions drop-down list (see Figure 3.12).

"click Rect"

Inactivated
ROI (green)
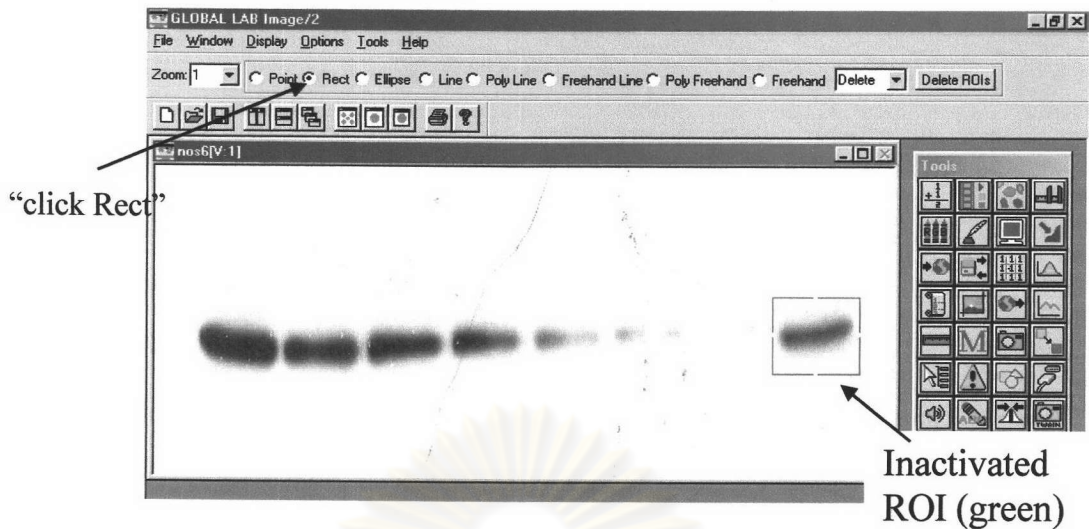
Figure 3.12. The image file of Western blotting outcome of COX-2 standard was inactivated through ROI tool menu.

4. In the rectangle ROI, click inside the ROI with the right mouse button. The newly activated ROI turns red (Figure 3.13). Previously inactivated ROI is green. After selecting, then activating by "click" on rectangle window again.
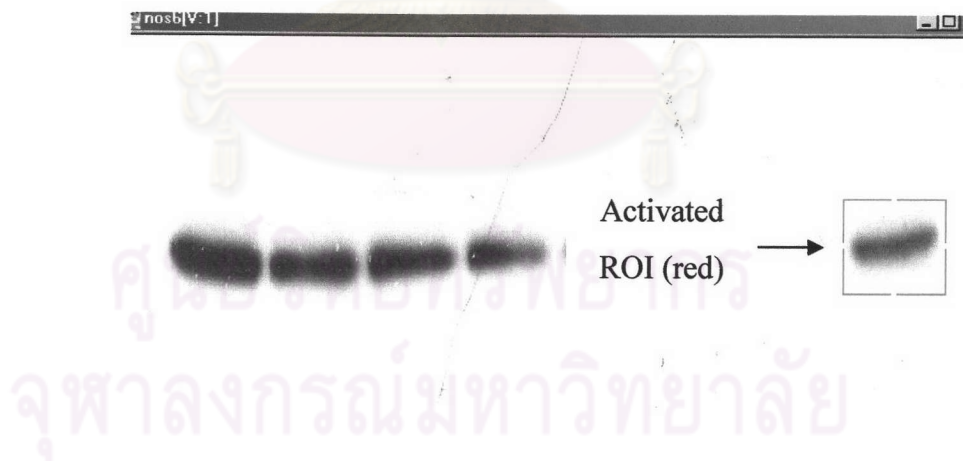
Activated
ROI (red)

Figure 3.13. The newly activated ROI turns red.

5. Open the Histogram Tool as described in detail in Appendix B (Figure 3.14). Click Add Histogram button to add a histogram to the graph (see Figure 3.15).
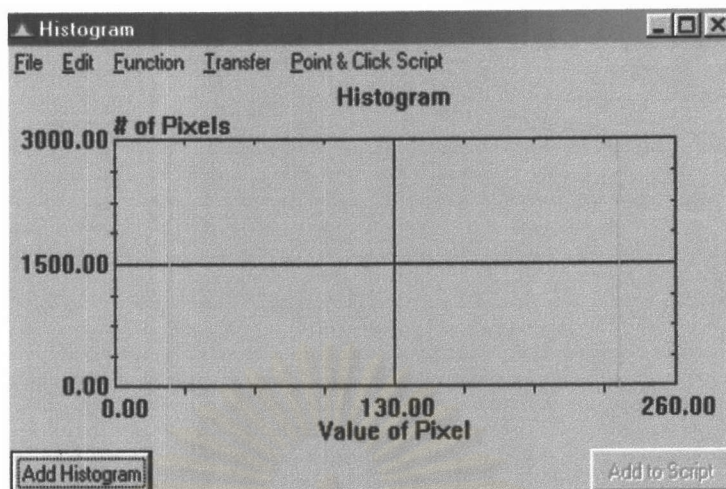
Figure 3.14. "Histogram Tool" was used for further analysis.

Add Histogram button
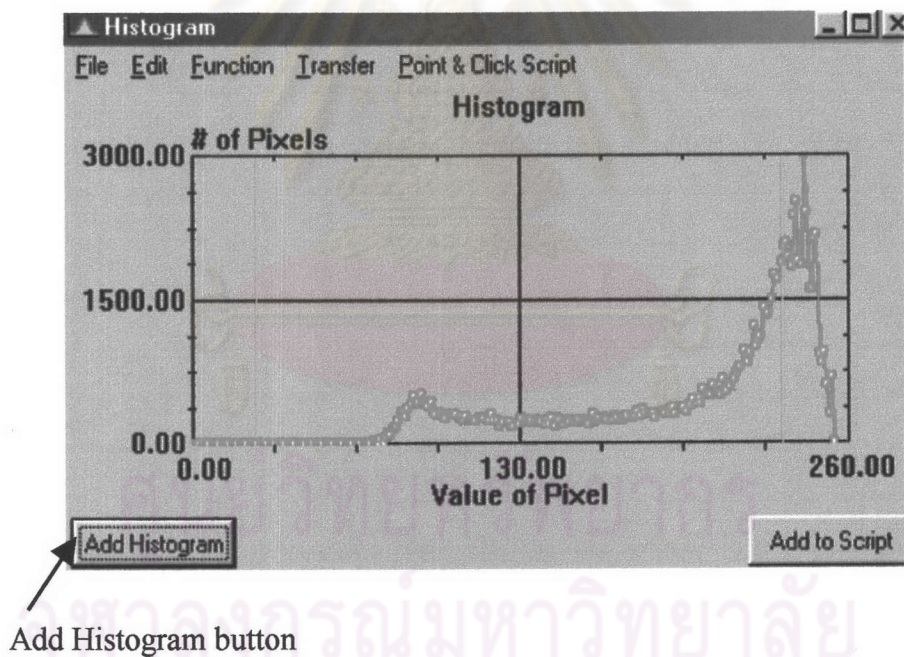
Figure 3.15. "Histogram" for analyzing digital image of COX-2 band

6. Select the Function Menu/Show statistics (Figure 3.16)
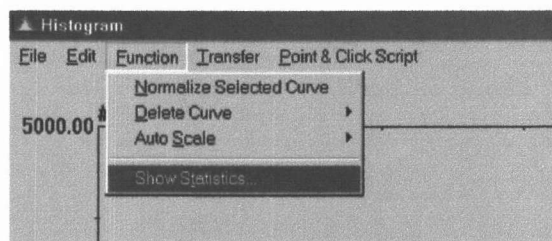
Figure 3.16. "Histogram" can perform the analysis.

The statistic values (Figure 3.17) are calculated with regard to the range of pixel values. Then record Mean value (the average pixel value in the selected range) for set specifying of the maximum threshold limits that appropriate for this image (COX-2 standard) in the Edge Finder Tool step.
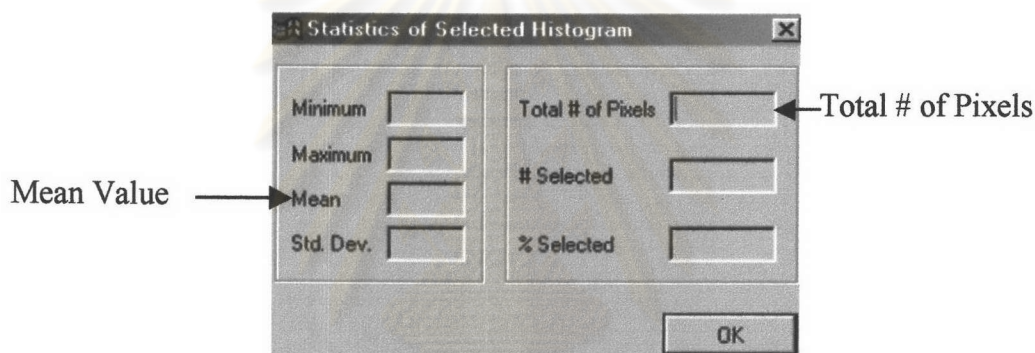
Figure 3.17. "Histogram" statistic calculation

7. Create a rectangle cover each COX-2 band (Figure 3.18).
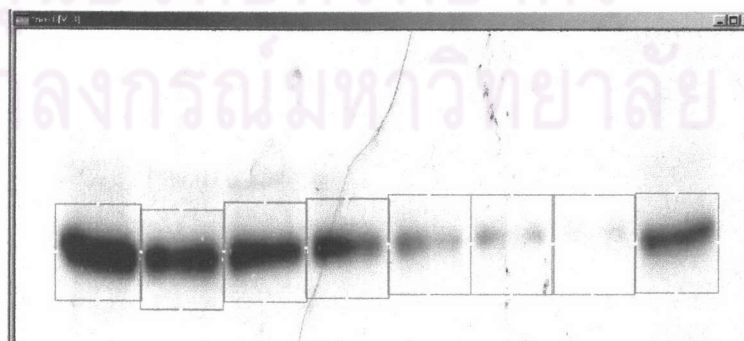
Figure 3.18. Demonstration of the created rectangles for every bands of COX-2 standards.

8. Open the Edge Finder Tool at mention below (Appendix B). Click in the viewport on the image COX-2 standard with left mouse button. Image COX-2 standard is now active viewport, then click input image (A) to load image COX-2 standard as input image (Figure 3.19). Select the first rectangle ROI that wants to generate edges by click inside ROI to activate it (turn red) (Figure 3.18). Select the Activate Threshold Controls (B) check box and use the Maximum (C) and Minimum (D) slider controls to adjust the threshold limits. The upper limit is equal Mean value of Histogram statistic in step 6 and lower limit is 0 value.
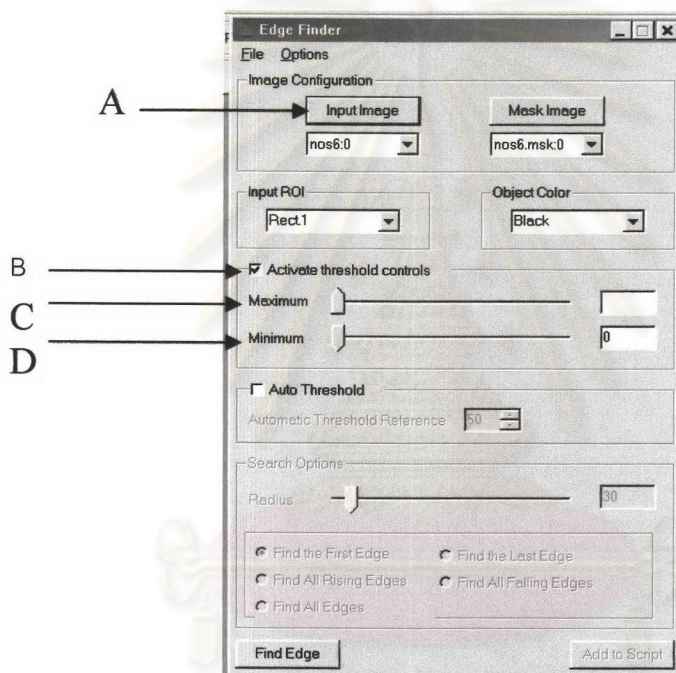
Figure 3.19. Edge Finder Tool

9. When all the setting are correct, click Find Edge button (E) to finds edges that within a first rectangle ROI (activated ROI) (Figure 3.20). Then activate another rectangle ROI and click Find Edge, repeat this step to all rectangles ROI (Figure 3.21).
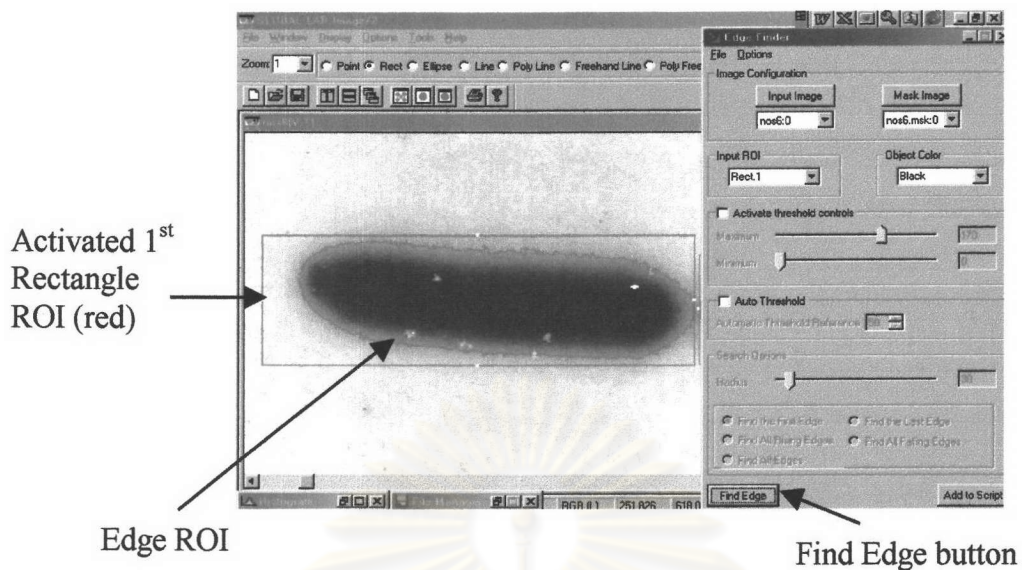
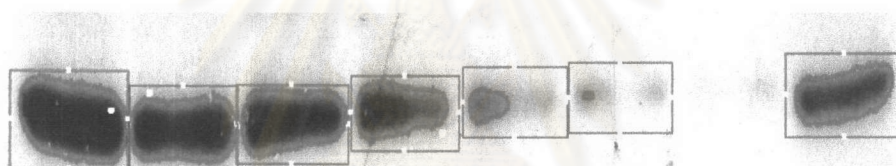Figure 3.20. Edge Finder tool was used to analyze for each activated rectangle.

Figure 3.21. The image file of Western blotting outcome of COX-2 standard was used and analyzed by Tool Edge that within all rectangles ROI.

10. Open the Histogram Tool (if one is not already open) as mention above in step 5. Activate first Edge ROI by click in it with the right mouse button (Figure 3.22).
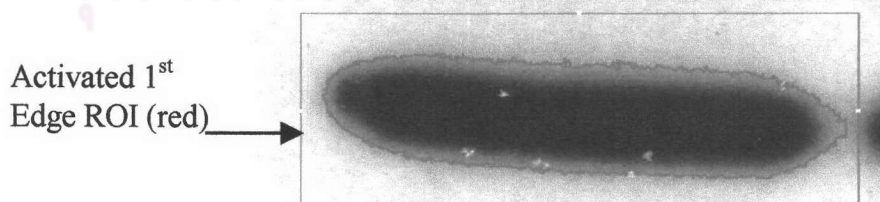

Figure 3.22. The activated Western blotting of COX-2 band after defining the edge

11. Click Add Histogram, then histogram was created using the first Edge ROI from image COX-2 standard (Figure 3.23).
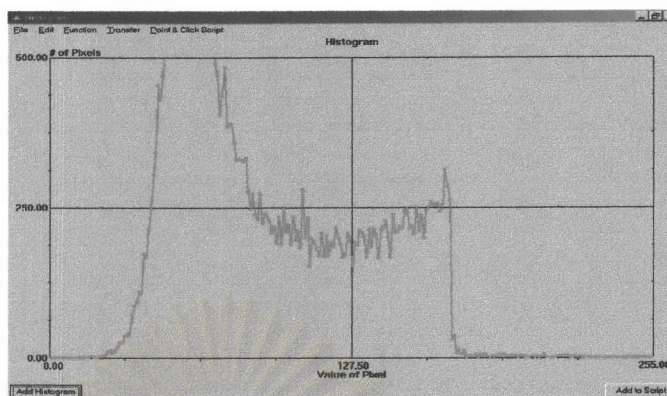

Figure 3.23. Histogram was created using the first Edge ROI from image COX-2 standard.

12. Select the Function Menu/Show statistics (Figure 3.16), then the result is a Total # of Pixels (Figure 3.17). After that the result of all selected histogram data (curve data and statistics data) can send to the Excel spreadsheet by select Transfer/DDE Transfer/Send Data to Excel (Figure 3.24).
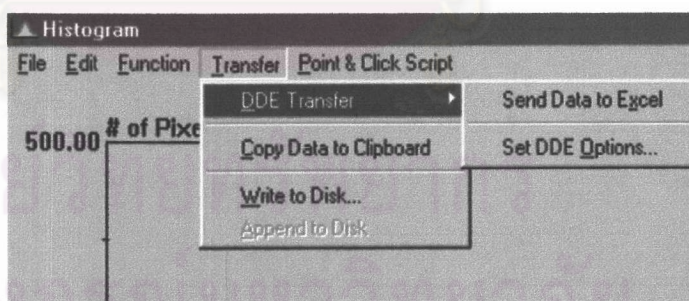

Figure 3.24. The histogram functional tool (Transfer), which was used for transfer histogram data (curve data and statistics data) to the Excel spreadsheet.

13. Repeat steps 10-12 for all Edge ROI from image COX-2 standard.

7.3 Standard curve of COX-2 protein.

COX-2 protein level was evaluated by Western blot analysis. An intense band developed on, from gel, transferred PVDF membrane, and exposed to ECL films was scaned (scanJet 6200C scanner, Hewlett Packard), and quantitated using an image analysis (GLI/2 software, area x density of band). To optimize the condition of COX-2 analysis, a standard curve generated by five bands from one gel made with serial concentrations of 0.0625, 0.125, 0.25, 0.5, 1 µg/10µl from dorsal skin fold tissues. It was found to be linear over a wide range (Figure 3.25). Positive control of 0.125 µg of protein was run in each blot to use as correcting factor for each run.

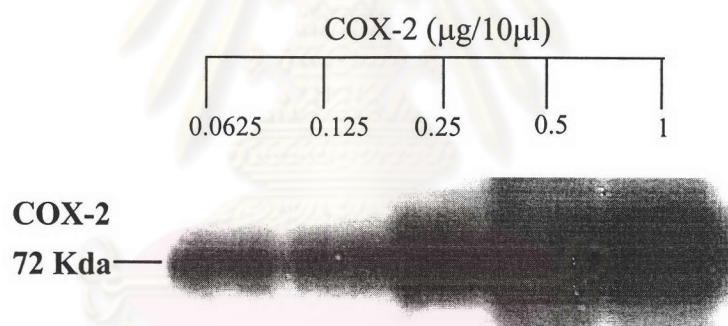The linear correlation equation is $y = 1412.5x + 267.67$ with highly significant ($p<0.001$) as shown in Figure 3.25.

Figure 3.25. The serial concentration (0.0625, 0.125, 0.25,0.5,1 µg/10µl) of COX-2 from one gel

Table 3.3. Number of pixels within the selected intensity range (#selected)

µg	0.0625	0.125	0.25	0.5	1
No.of pixels (#selected)	273	362	737	1099	1604

In each Western blot experiment, 10 µg of protein per well were used. Sample of HMW marker, positive control (0.125 µg/10µl), and tissue extract from each sample were together applied for every experiments. Then image analysis were used for evaluate the number of pixels for each COX-2 band as described previously. Calculation of COX-2 protein in each sample was evaluated from the standard equation of $y = 1412.5x + 267.67$ as shown in Figure 3.26.
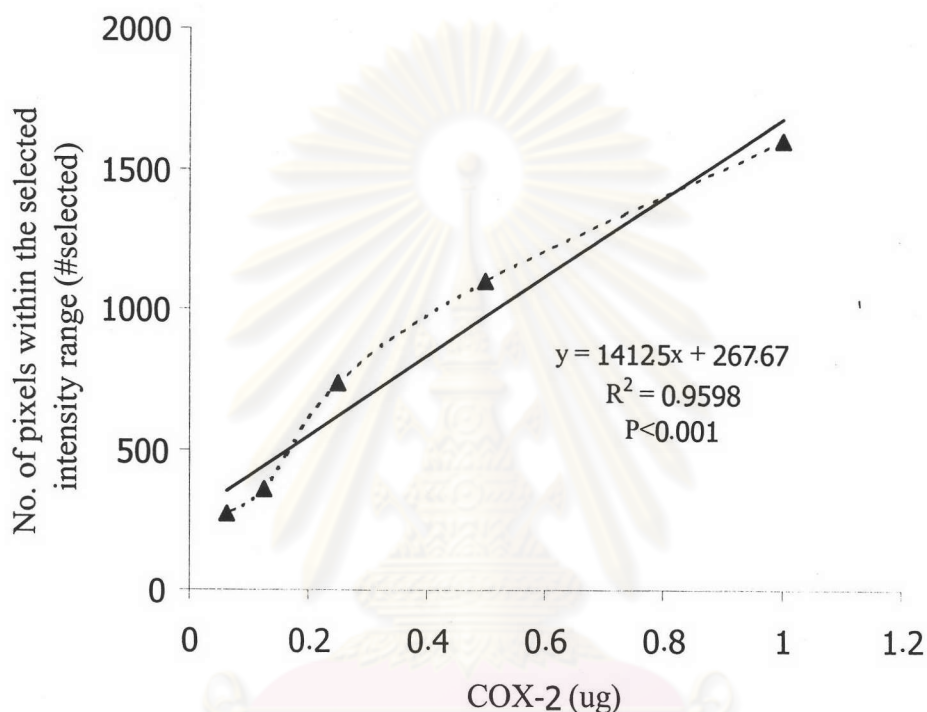

Figure 3.26. Standard curve for optimized condition COX-2 protein level. Correlation between serial concentration of COX-2 standard (µg) and number of pixels within the selected intensity range (#selected)

Histological Examination

At the end of the experiment, the specimen of tissues in area within dorsal skin-fold chamber were fixed in buffered formalin. The section was stained with Hematoxylin and Eosin (H&E) staining. Therefore, the tumor growth were confirmed by the Pathologist (HI-TECH PATH. LAB).

Data analysis

All data were presented as means and standard errors of mean (SEM). For comparison among groups of animals, one-way analysis of variance (one-way ANOVA) was used and the differences in pairs of means among groups were made by LSD test. The relationships between capillary density and COX-2 and VEGF, and between time coures and neocapillary density, COX-2, and VEGF and were made by linear regression and Model fit curve. If the statistical probability (p-value) was less than or equal to 0.05, the differences were considered to be statistically significant.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย