3. MATERIAL AND METHODS

3.1 STUDY DESIGN

This study was a cross-sectional study for microbiological examination of samples from animals that were being examined in a parallel study at the farm level.

3.1.1 Study sites

A standard slaughterhouse belonging to a pork production company in Chiang Mai was selected. The pigs slaughtered in this slaughterhouse came from over 40 contract farms located in the Chiang Mai and Lumphun provinces. The pig slaughtering and pork processing were commercially performed for local consumption at a rate of approximate 120 - 140 pigs per day.

3.1.2 Sample type and laboratory investigation

According to the *Salmonella* study, along the pork chain, the slaughter pigs and carcasses sampled for this study were the same ones, which were investigated in a sister study at the farm level. The slaughter pigs were re-identified by ear tattoo and spray marking at the slaughterhouse. During the slaughtering process, tag numbers used to identify the pigs were secured on the forelegs of the carcasses. From each individual pig, one fecal, one lymph node and two-carcass swab samples (before chlorinated-water spray and after overnight chilling) were collected for *Salmonella* isolation. One muscle sample was also collected for serological investigation.

Salmonella isolation was performed following ISO 6579 (2002) and Sifin® Enteroclon Anti-Salmonella Antigens (Slide agglutination methods) were used for *Salmonella* serotyping. For serological testing, the commercial ELISA test kit SALMOTYPE® Pig LPS ELISA was used.

3.2 SAMPLE SIZE DETERMINATION

The Win Episcope 2.0 was used for sample size determination. A population estimate of 30,000 pigs from all contract farms and prevalence of *Salmonella* of 69.5 % in pre-slaughter pigs in Chiang Mai Province (ranging from 50-83%) (Patchanee *et al*. 2002) was used. At a 95% level of confidence and 8% error rate, the calculated sample size for this study was 128 pigs. However, 181 pigs were actually selected from the 21 farms (17 open- and 4 closed-house raising systems). The sample sizes from these two systems were 141 and 40 pigs respectively.

3.3 THE SLAUGHTERING PROCESS

Upon arrival, pigs were immediately sprinkler-showered with potable water and rested for 1-2 hours. They were then transferred to the slaughter line, stunned using low voltage electrical tongs (110 - 180 Volts) for less than 15 seconds. The stunned pigs were secured to an overhead conveyor rail by a chain looped around one of the hind angles. By cutting the main blood vessels of the neck using a sharp knife, the animals were immediately bled. Combined scalding and dehairing of pigs was done for 1.30 – 3.0 min. in a scalding tank (kept at temperatures between 62 and 65°C). The final dehairing was manually performed using a sharp knife.

After dehairing, the slaughter pigs were given a pre-evisceration wash by manual hosing with potable normal water. A singeing or flaming procedure was not implemented in this slaughterhouse. Slaughtered pigs were secured to an overhead conveyor chain by hooking the hind legs. Evisceration involved three separate tasks (de-bunging, slitting the belly open and gut removal), all of which were performed by the same operative. De-bunging or detachment of the rectum was completed prior to opening the belly. The connective tissues joining the bung and viscera to the carcasses were cut. The diaphragm, heart, lungs, and some part of trachea were manually removed together as part of the pluck set, along with the digestive tract. At this stage, the head was removed.

The carcasses were manually cut along the midline from the hind to the fore using a power-splitting saw. Thereafter, the kidneys, spinal cord, and fascia were removed.

The carcasses were finally hose-washed with normal potable water and sprayed with cold (5 - 10°C), chlorinated water (50 - 100 ppm) to lower the bacterial load before shock freezing (-18 to -20°C for approximate 2 hrs) and cold storage overnight in the chilling room (≤ 4°C). Further cutting and processing were performed the next day after overnight chilling.

3.4 COLLECTION OF SAMPLES

3.4.1 Fecal samples

Immediately after evisceration, at least 25 g of intestinal contents was taken from the colon of each sampled pig using disposal gloves, kept in plastic bags and refrigerated (4°C) in an icebox. The samples were brought to laboratory within 4 hours of collection and tested for *Salmonella* with 24 hours post collection.

3.4.2 Meat and lymph node samples

Ten grams of diaphragmatic muscle was collected for ELISA testing. Meat juice was harvested by freezing and then thawing of the muscle samples. This was done in the laboratory.

At least 25 g of intestinal lymph nodes were collected from the same carcass. Both meat and lymph node samples were collected using sterile tools. Each sample was collected in a plastic bag and kept refrigerated at 4°C in an icebox container. Time of sample delivery and laboratory procedure was the same as described in section 3.4.1.

3.4.3 Carcass swabs

The carcass swabs were taken on the carcass surface at two stages: (1) prior to spraying the carcass with chlorinated-water and (2) following overnight chilling.

Based on the Commission Decision of the European Communities (The Commission of the European Communities, 2001), four carcass swabs were taken (Figure 7) pooled into one sample. Because the heads were removed before swab taking, the lowest part of the neck was swabbed instead of the jowl in this case. The samples were then kept in a separate icebox and transported to the laboratory within a few hours after collection (the same procedure as the other samples).

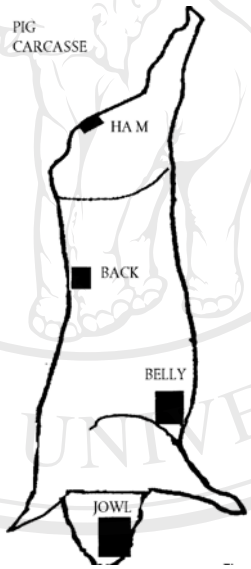

Figure 7: Swab sampling sites on the carcass
(Source: Official Journal of the European Community 471/2001)

Cotton swabs were moistened in sterile normal saline prior to sampling. Each of the sites was at least 100 cm^2. Moistened swabs were rubbed in the following motion: vertically, then horizontally, then diagonally for not less than 20 seconds across the entire surface site. The pooled samples were stored in a bottle of 50 ml Buffered Peptone Water before transportation to the laboratory.

3.4.4 Overall numbers of samples

Overall, 723 samples were taken for *Salmonella* investigation, i.e.181 fecal samples, 181 lymph nodes, and 181 carcass swabs taken before carcasses were washed with chlorinated-water. The rest, 180 carcass swabs, were taken after the carcass was chilled overnight. In addition to the lab samples, 181 diaphragm muscles were collected for ELISA testing.

3.5 LABORATORY PROCEDURES

3.5.1 Conventional culture: ISO 6579 (2002)

3.5.1.1 Salmonella isolation

- *Sample preparation*

Twenty-five grams of fecal samples were mixed with 225 ml Buffered Peptone Water (BPW; Merck KGaA, Germany). The lymph node samples were each put into 70% alcohol and flamed for a few seconds to eliminate superficial contamination, and thereafter cut into small pieces with a sterile scalpel. Thereafter, 10 g of the sample was transferred into a stomacher bag filled with 90 ml Buffered Peptone Water. The muscle samples were also cut into small pieces using sterile scalpels or blades and then kept separately in plastic bags and kept frozen.

- *Pre-enrichment*

A pre-enrichment medium was used to resuscitate any stressed microorganisms and enhance their growth. The medium of choice recommended for *Salmonella* resuscitation is the highly nutritional and non-selective medium, Buffer Peptone Water (BPW; Merck KGaA, Germany). After transferring 25 g of samples into a stomacher bag, 225 ml of Buffer Peptone Water was added and then stomachered for 2 minutes. All samples were incubated at 37°C for 18–24 hrs.

- *Selective enrichment*

The pre-enrichment broth was mixed and 0.1 ml was transferred to 9.9 ml pre-warmed Rappaport Vasiliadis (RV; Merck KGaA, Germany) enrichment broth, which was incubated at 42°C for 24–48 hrs. One ml of the broth was also transferred to 9.0 ml of Tetrathionate (TT; Merck KGaA, Germany) broth for the secondary selective enrichment. It was incubated at 37°C for 24 hrs.

- *Selective solid media*

A loop of material from the RV broth was transferred and spread onto the surface of a Xylose-lysine-tergitol 4 (XLT4; Merck KGaA, Germany) agar. The second agar of choice, Brilliant Green Phenol Red Lactose Sucrose (BPLS; Merck KGaA, Germany) agar, was used for growing the isolated colonies. The plates were incubated in an inverted position at 37°C for 18–24 hrs. After incubation, the plates were checked for growth of typical *Salmonella* colonies. The latter have a black center and a lightly transparent zone of reddish color on XLT4 and reddish color and a translucent colony on BPLS.

When no typical colonies were found after 24 hrs of incubation, a loop of both enrichment broths was plated out again on XLT4 and BPLS agar and then incubated for another 24 hrs at 37 °C.

- *Confirmation*

Suspected colonies were streaked on the surface of pre-dried nutrient agar plates and incubated at 37±1 °C for 24 ± 3 hrs, in a manner that allowed the isolated colonies to develop. Up to five colonies per plate were purely cultured and used for biochemical and serological confirmation. Both methods were performed and interpreted as described in subsection 5.5.1.2 and 5.5.1.3.

3.5.1.2 Biochemical confirmation

The pure colonies after incubation on nutrient agar were picked up and inoculated into Triple Sugar Iron (TSI; Merck KGaA, Germany) slant, Voges-Proskaur (VP; Merck KGaA, Germany) broth, Motile-indole-lysine (MIL; Merck KGaA, Germany) broth and Urea (Urea; Merck KGaA, Germany) slant. All inoculated biochemical media were incubated at 37°C for 18-24 hrs, with the exception of VP that was incubated for 48 hrs.

The biochemical confirmation followed the results reactions. In the case of *Salmonella*, the reaction would present the following appearances.

1. Glucose : Positive (+)
2. Gas : Positive (+)
3. Lactose : Negative (-)
4. Sucrose : Negative (-)
5. H_2S : Positive (+)
6. Urease : Negative (-)
7. LDC : Positive (+)
8. VPR : Negative (-)
9. Indole : Negative (-)

3.5.1.3 Serological confirmation

The serological confirmation of *Salmonella* antigens was performed by slide agglutination testing, according to the commercial product (SIFIN®, Germany). All isolates from each type of sampls were tested using the following antisera:

a) *Salmonella* Polyvalent I (A-E) and II (F-67)
b) *Salmonella* Somatic (O) Group A, B, C, D and E, and
c) *Salmonella* Flagella (H) Antisera set

The sequences of the serological testing are depicted in Figure 8.

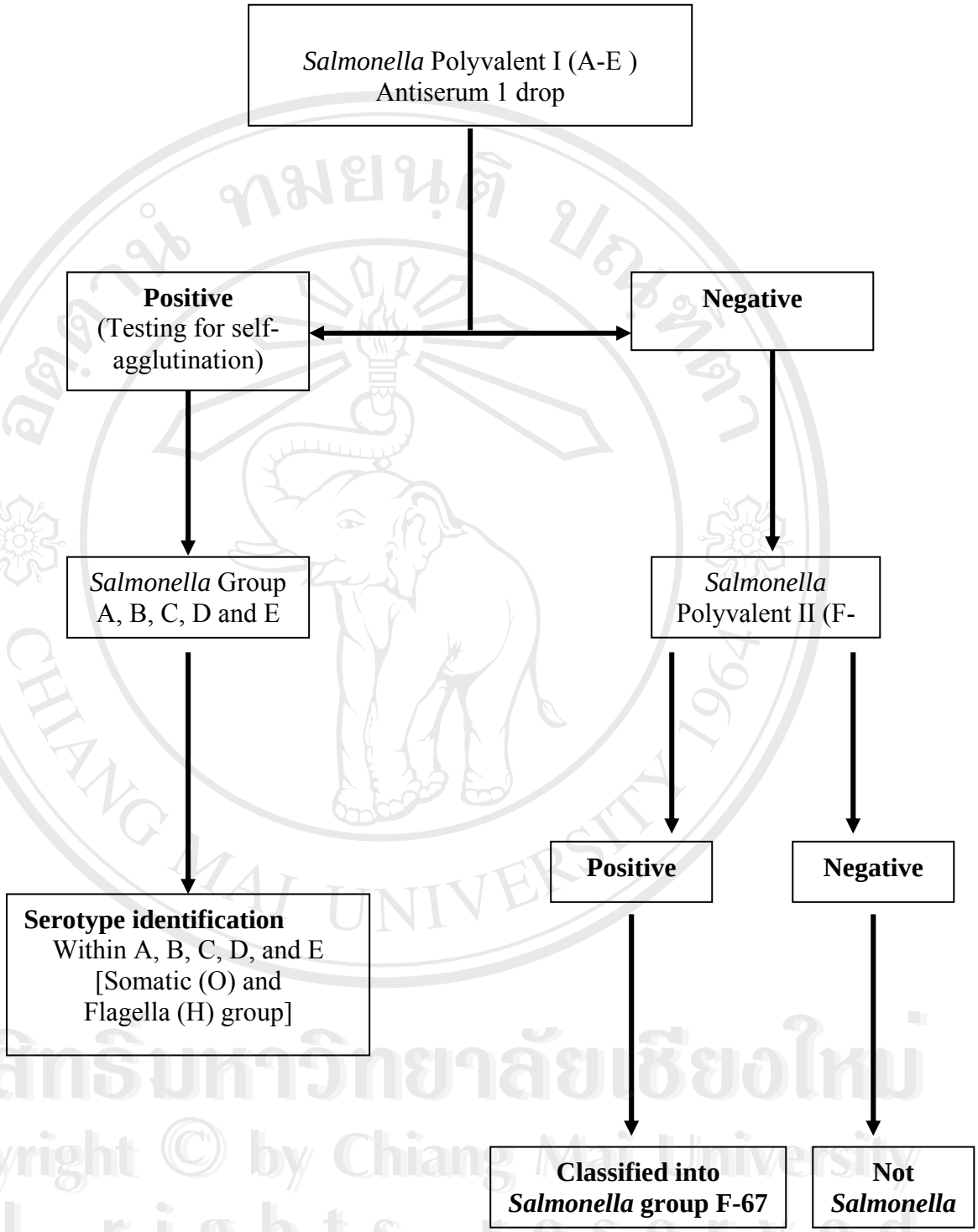

Figure 8: *Salmonella* Serotyping flow chat (Slide agglutination test)

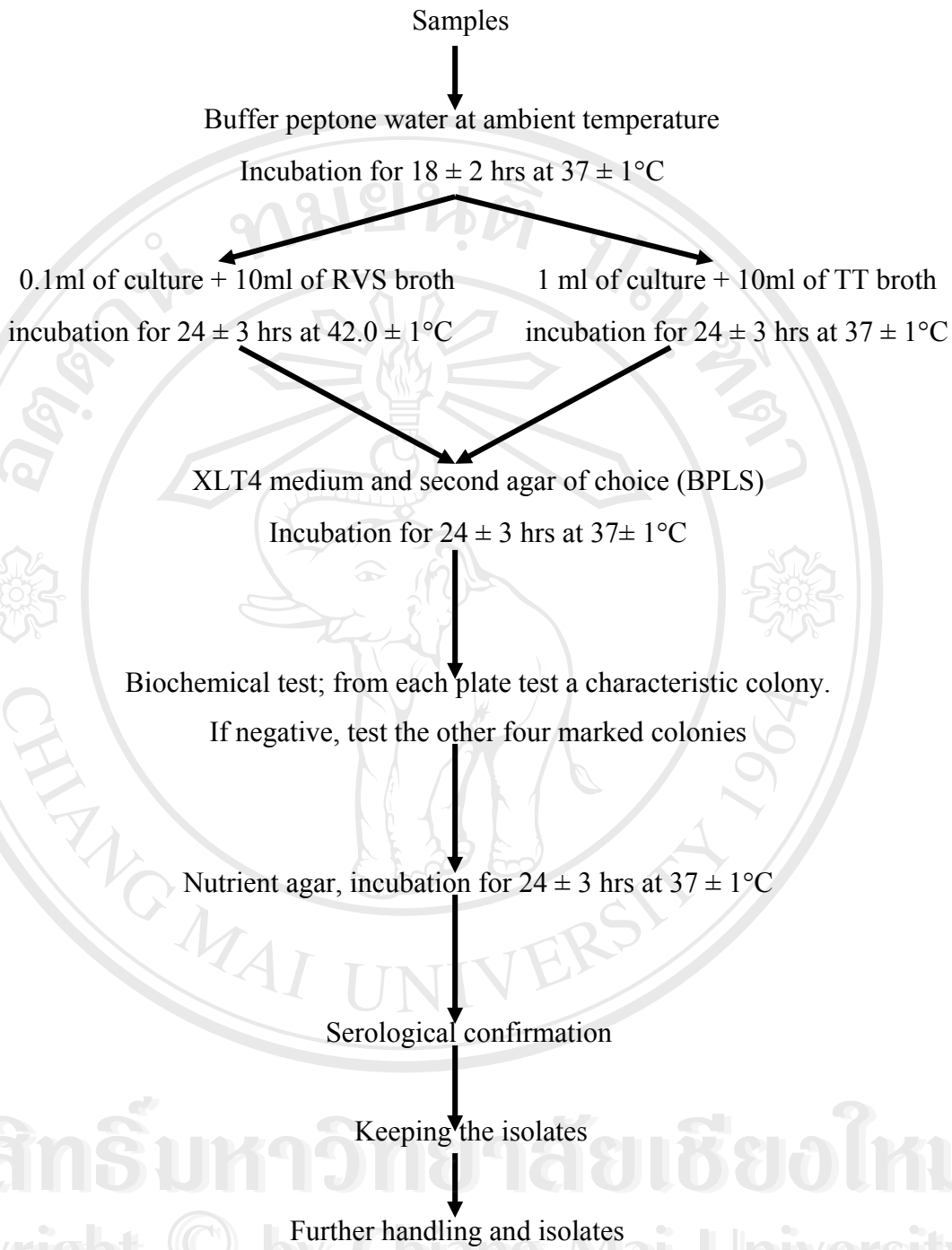

Figure 9: Overall summary of sample handling and *Salmonella* identification procedure

3.5.2 Serological testing: SALMOTYPE® Pig LPS ELISA

3.5.2.1 Usage of the test

An enzyme immunoassay (ELISA) for the detection of specific antibodies against *Salmonella* in pork meat juice was used. In this study the diaphragmatic muscle was used and meat juice was harvested by thawing the frozen pork meat. The steps following were those given by the manufacturer, Labor Diagnostik Leipzig, Germany (Figure 10). The test result was the optical density produced by the sample relative to the optical density of the positive reference sample (O.D. %). Negative or positive result was interpreted following the different cut-off values of O.D. %. As recommended by the manufacturer, interpretation was as follows.

3.5.2.2 Result interpretation

- Cut-Off values for samples (serum, meat juice, plasma);
  ≥ 40 OD% positive
  20 OD% - < 40 OD% weak positive
  10 OD% - < 20 OD% doubtful (positive)
  < 10 OD% negative

- Cut-Off values of samples for categorization of herds according to monitoring programs:
  ≥ 40 OD% or ≥ 20 OD% are positive depending on national regulations
  For the assay to be valid, the P/N-ratio between the Positive Control Serum 1 (P) and the Negative Control Serum (N) should be greater than 4.0.

3.5.2.3 Flow chart of the steps used in SALMOTYPE® Pig LPS ELISA

The instruction of the test is presented in the Figure 10.

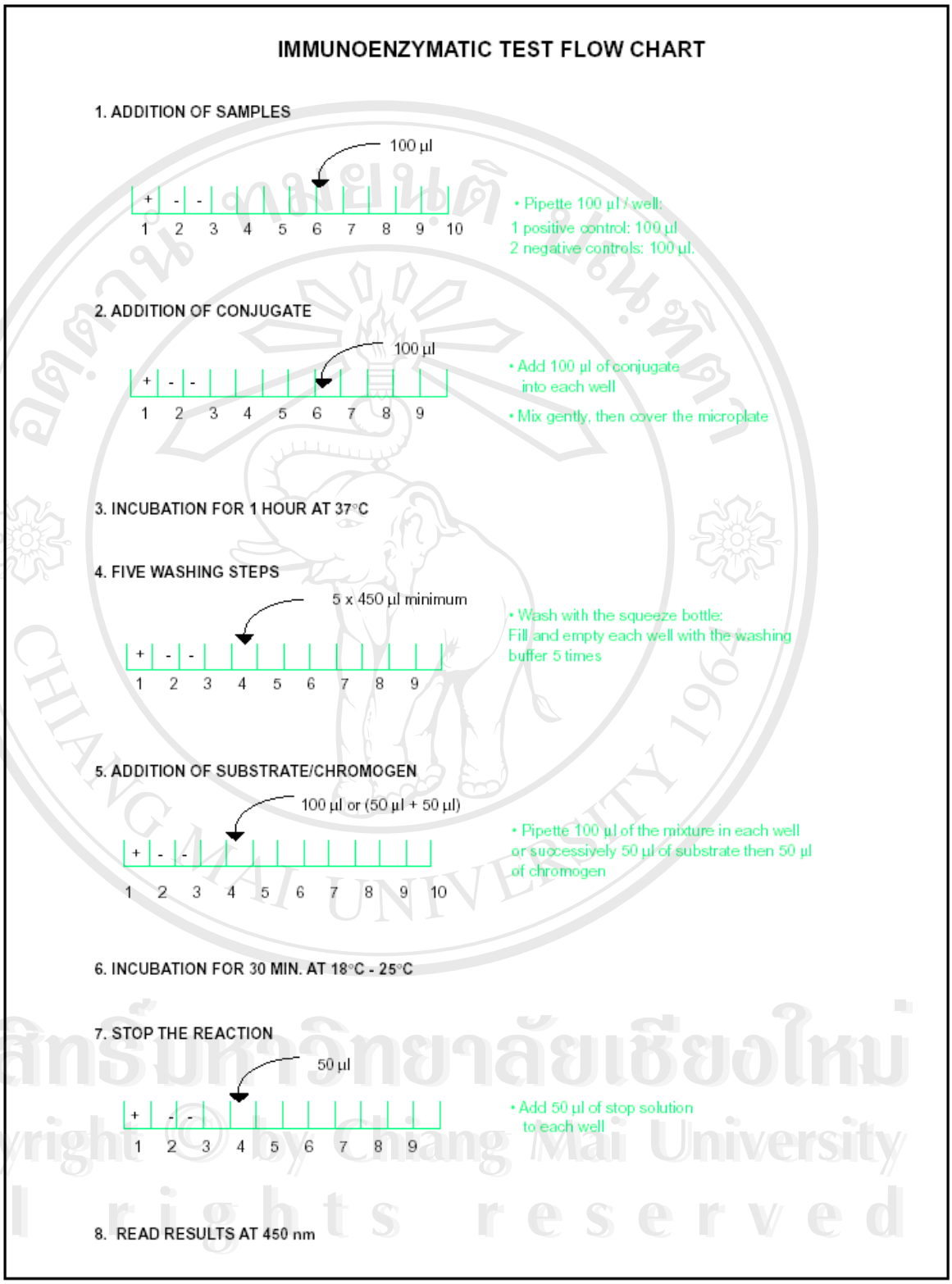

Figure 10: Flow chart of SALMOTYPE® Pig LPS ELISA (Labor Diagnostik, Leipzig)

3.6 DATA MANAGEMENT AND STATISTICAL ANALYSIS

Data from conventional *Salmonella* culture was entered into a Microsoft Excel database and error checks done. In order to reach thesis objectives, data analysis was conducted following these performances.

(1) Using the Excel calculation program, sample prevalence of *Salmonella* and 95% confidence intervals (CI 95%) was calculated.

(2) Distribution of *Salmonella* serotypes in various types of samples and from different farms of origin was conducted using the pivot table in Microsoft Excel.

(3) Win Episcope 2.0 was used for calculation of sensitivity and specificity of the ELISA test, using bacteriological culture (lymph node- and fecal culture results) as the golden standard.

(4) Kappa statistics was used for assessment of agreement between two different methods of *Salmonella* isolation, conventional culture of fecal samples and commercial ELISA test using Epi Info 2002. According to Dahoo *et al.* (2003), the criterion of kappa statistics was categorized into the following:

Kappa value <0.2: slight agreement
0.2- 0.4: fair agreement
0.4-0.6: moderate agreement
0.6-0.8: substantial agreement
>0.8: almost perfect agreement