MASS SENSITIVE IMMUNOSENSOR FOR MICROALBUMIN DETERMINATION

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Thesis
Entitled

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MASS SENSITIVE IMMUNOSENSOR FOR MICROALBUMIN DETERMINATION

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ABSTRACT

Albumin is normally found in the blood circulation system and is filtered by a functional unit of kidney. Minute amounts of albumin can appear in urine as a consequence of kidney damage, so called “Microalbuminuria”. A sensitive and reliable analytical method is required for determining of microalbumin in urine in order to identify renal dysfunction.

A mass sensitive immunosensor based on quartz crystal microbalance (QCM) was employed to detect microalbumin concentration in urine samples. The active surface of this immunosensor was prepared by coating of protein A on silver electrode surface of 12 MHz AT-cut quartz crystal. Then, anti-human albumin was immobilized on protein A layer of crystal surface. The sample containing albumin was applied to this active surface for detection of specific immunological binding. Downward shift of oscillation frequency was monitored as an indicator of binding event.

The optimal concentration and coating time of protein A to prepare the surface for immobilization of anti-human albumin was 1.0 mg/ml for 2 hours at room temperature. The optimal immobilization concentration of anti-human albumin on protein A layer was 0.05 mg/ml for 2 hours at room temperature.

Microalbumin was detected by a decrease of oscillation frequency in the range 100 - 5000 ng/ml in logarithmic dose response curve. The detection limit of microalbumin determination was 9.5 ng/ml. There was no statistically significant difference of results from 64 urine samples ($p>0.01$) when determined with this immunosensor and with standard immunoturbidimetric method.

This immunosensor can be kept in a moisture chamber at 4 ℃ up to 2 months without reduction of their performance. This was satisfactory from analytical perspectives, and therefore this method should be suitable to monitor microalbumin in urine samples.

KEY WORDS: MICROALBUMIN/PIEZOELECTRIC IMMUNOSENSOR/ BIOSENSOR/ QUARTZ CRYSTAL/ IMMUNOTURBIDIMETRY

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แมสเซ็นซิทิฟอิมมูโนเซ็นเซอร์สำหรับการตรวจวัดไมโครอัลบูมิน (MASS SENSITIVE IMMUNOSENSOR FOR MICROALBUMIN DETERMINATION)

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บทคัดย่อ

อัลบูมินเป็นโปรตีนที่ปกติพบในระบบไหลเวียนโลหิต ภาวะที่พบอัลบูมินจำนวนน้อยๆ ออกมาในปัสสาวะอาจแสดงถึงความผิดปกติของไตซึ่งเรียกภาวะนั้นว่า “ไมโครอัลบูมินยูเรีย” ซึ่งต้องการวิธีตรวจวิเคราะห์ที่มีความไวและน่าเชื่อถือในการตรวจวัดไมโครอัลบูมินในปัสสาวะเพื่อใช้ในการช่วยวินิจฉัยโรคไต

การพัฒนาระบบตรวจวัดไมโครอัลบูมินในปัสสาวะโดยวิธีทางอิมมูโนเซ็นเซอร์ ใช้เทคนิคของควอทซ์คริสตัลไมโครบาลานซ์นำมาตรวจวัดปฏิกิริยาระหว่างแอนติเจนและแอนติบอดี้ การเตรียมพื้นผิวอิเล็กโทรดรดเงินของควอทซ์คริสตัลชนิด AT-cut ที่มีความถี่พื้นฐาน 12 เมกะเฮิร์ทซ์ สำหรับการตรวจวัดอัลบูมินนั้นทำได้โดยการตรึงแอนติบอดี้ที่จำเพาะต่ออัลบูมินที่ผิวอิเล็กโทรดเงินด้วยโปรตีนเอ โดยจะทำการวัดค่าความถี่ของควอทซ์คริสตัลที่ลดลงเป็นตัวบ่งชี้ปฏิกิริยาระหว่างแอนติบอดี้และอัลบูมินหลังจากใส่ตัวอย่างที่มีอัลบูมินแล้ว

ความเข้มข้นของโปรตีนเอและเวลาในการทำปฏิกิริยาที่เหมาะสมสำหรับการตรึงแอนติบอดี้คือ 1.0 มิลลิกรัมต่อมิลลิลิตรและ 2 ชั่วโมงที่อุณหภูมิห้อง ความเข้มข้นของแอนติบอดี้และเวลาในการทำปฏิกิริยาบนพื้นผิวเงินที่มีโปรตีนเอคือ 0.05 มิลลิกรัมต่อมิลลิลิตรและ 2 ชั่วโมงที่อุณหภูมิห้อง

หลังจากได้สภาวะที่เหมาะสมในการตรวจวัดไมโครอัลบูมินแล้วมีการคำนวณค่าขีดจำกัดในการตรวจวัดโดยวิธีนี้คือ 9.5 นาโนกรัมต่อมิลลิลิตร เมื่อนำตัวอย่างปัสสาวะจำนวน 64 ตัวอย่างมาทำการตรวจวัดหาค่าไมโครอัลบูมินโดยวิธีอิมมูโนเซ็นเซอร์แล้วนำไปเปรียบเทียบกับค่าที่ได้โดยวิธีที่ใช้ในห้องปฏิบัติการในโรงพยาบาลทั่วไปพบว่าทั้งสองวิธีมีความสัมพันธ์ที่ดีและไม่มีความแตกต่างกันในทางสถิติ ($p>0.01$)

ระบบอิมมูโนเซ็นเซอร์นี้สามารถเตรียมพื้นผิวที่มีโปรตีนเอและแอนติบอดี้เก็บไว้ได้ไม่น้อยกว่า 2 เดือนในอุณหภูมิ 4 องศาเซลเซียส โดยที่ความสามารถไม่ลดลง วิธีนี้เหมาะสมที่จะใช้ในการตรวจวัดไมโครอัลบูมินในปัสสาวะ

85 หน้า

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LIST OF ABREVIATIONS

%	=	percentage
^{o}A	=	amstrong
Ab	=	Antibody
ADA	=	The American diabetes association
ADH	=	Anti-diuratic hormone
Ag	=	Antigen
BSA	=	Bovine serum albumin
BUN	=	Blood uria nitrogen
^{o}C	=	degree celcius
CCr	=	Creatinine clearance
CKD	=	Chronic kidney disease
cm^2	=	centimeter square
Da	=	Dalton
ELISA	=	Enzyme-link immunosorbent assay
F_0	=	Fundamental frequency
Fab	=	Fragment antigen binding
Fc	=	Fragment crystallisable
g	=	gram
g/cm^2	=	gram per centimeter square
g/cm^3	=	gram per centimeter cube
GFR	=	Glomerular filtration rate
h	=	hour
HCl	=	Hydrochloric acid
HIV	=	Human immunodeficiency virus
HPLC	=	High performance liquid chromatography
Hz	=	hertz
Hz/day	=	hertz per day
IgG	=	Immunoglobulin G

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LIST OF ABREVIATIONS

(continued)

INM	=	Immunonephelometry
kDA	=	kilo Dalton
KCl	=	Potassium chloride
KH_2PO_4	=	Potassium phosphate
L	=	liter
MHz	=	megahertz
μl	=	microliter
mg	=	milligram
mg/ml	=	milligram per milliliter
min	=	minute
ml	=	milliliter
ml/min	=	milliliter per minute
NaCl	=	Sodium chloride
Na_2HPO_4	=	Sodium phosphate dibasic
NaOH	=	Sodium Hydroxide
ng/ml	=	nanogram per milliliter
NKF	=	The national kidney foundation
PCR	=	Polymerase chain reaction
QCM	=	Quartz crystal microbalance
ELISA	=	Enzyme Linked Immunosorbent Assay
PBS	=	Phosphate buffer saline
psi	=	pound per square inch
RIA	=	Radioimmunoassay
RT	=	Room temperature
SAW	=	Surface acoustic wave
S.D.	=	Standard deviation
TSM	=	Thickness shear mode

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CHAPTER I

INTRODUCTION

Chronic kidney disease is a world-wide public health problem, with increasing incidence and prevalence, poor outcomes, and high medical expense. The results of chronic kidney disease dose not only kidney failure but also include complication of decreasing kidney function and cardiovascular disease. There is portent growth in chronic kidney disease among the world's population. The investigation by WHO has shown the prevalence of kidney failure was 300-500 million of patients worldwide and has risen steadily for over 30 years. The most patients with end-stage of kidney disease will end up on dialysis because of limited kidney transplantation supply (1, 2).

Recent studies, kidney disease can be delayed or prevented if it is diagnosed and managed at early state. Therefore, early intervention may retard its progression to kidney failure and reduce the complications associated with decreased kidney function. The early diagnosed in these patients may play a role in treatment cost reduction including the cost of dialysis.

In the new-fashioned, trend to go to health check-up more frequently than the old days. However, using the present screening kidney function tests, the number of new patients register to kidney transplant program in US has still increased constantly in the past 20 years and its forecast to continually increase (3). Since, a new improved screening process will be needed.

There are many metabolic functions and chemical interactions to be evaluated through laboratory tests of kidney function. Kidney function tests look at whether the kidney is failing by measuring the waste products passed out in the urine and those left behind in the blood. The laboratory markers are very crucial for detecting injured kidney function. Glomerular filtration rate (GFR) is promising index, as gold standard method to evaluated kidney function. The method based on measuring of inulin, radio-

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labeled substance or iohexol, anyhow it is to complex and laborious for routine clinical laboratory. In clinical practice, the routine tests for assessing the kidney function are the blood creatinine, blood urea nitrogen (BUN) and uric acid (4, 5). However these tests have poor sensitivity as clinical marker in routine tests for evaluated kidney function (6, 7). A new marker is needed to test kidney function that more sensitive and precise in diagnosed kidney function.

Proteinuria refers to an excess of protein excretion in urine. When kidneys are damaged, protein leaks into urine. A simple test can be done to detect protein in urine. Persistent protein in the urine is an early sign of chronic kidney disease. In 1836 Richard Bright was the first physician to show clearly that the test of albumin was demonstration of kidney disease (8). In 1980 Viberti and Mogensen showed that even a slightly elevated urine albumin concentration, not detectable with urine dipstick test, was predicted in diabetic nephropathy (9, 10). The American Diabetes Association and The National Kidney Foundation (NKF) have been recommended the routine screening for microalbumin as a marker for early detection of diabetic nephropathy and chronic kidney disease (1, 11, 12).

Radio-based and fluorescence-based immunoassays are standard methods to quantification of urinary proteins. Radio-based immunoassay (RIA) has many advantages, such as simplicity, sensitivity and handling and efficacy, however the health hazards, costs of handling radioisotopes, and the short stability time associated with RIA has stilled to be a problem. Fluorescence- based immunoassays also have high sensitivity but also the shortcoming of multiple washing steps, the need for expensive secondary reagents such as materials labeled with a fluorescent probe which is excited and fluoresces in the visible region of the spectrum. In addition, this places a limitation on its use with some materials, such as low molecular weight hormones and proteins, which in some cases are perturbed in the interaction by the presence of the fluorescent label (13-15). HPLC can not completely discriminate albumin from other urinary proteins if it is not combined with special detection processes (16-18). Immunonephelometry (INM) and immunoturbidimetry, a newer technique with full automation that has became the major method for protein quantification, requires artificial facilities and fixed matched reagents and offers suboptimal precision, all of which limit its clinical applications (19-21). Therefore, a fast, sensitive, label-free, and

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easy-to-manipulate technique for urinary protein quantification is needed for clinical use.

Piezoelectric quartz crystal microbalance (QCM) immunosensors, the measurement of the frequency change of the quartz before and after immunological reaction was commonly successfully develop for clinical chemistry application, because of the inherent specificity of antigen–antibody combinations and high sensitivities of physical transducers (22-24). The QCM is a promising candidate for the detection of urine protein is simple and inexpensive. According to the high sensitivity and specificity, easy to use and rapid detection for microalbumin using QCM immuno-based biosensor technology will be studied.

Research Objectives

1. To develop the rapid detection method for microalbumin by using the specific immuno-based biosensor system.
2. To evaluate the sensitivity and specificity of specific immuno-based biosensor system for detection of microalbumin.
3. To compare the methods for microalbumin detection in urine sample between mass sensitive immunosensor and conventional system

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CHAPTER II
LITERATURE REVIEW

2.1 Kidney

The kidneys are paired, bean-shaped organs, which lie retroperitoneally and measure approximately 11 cm in length. Their anatomical relations are shown in Figure 2.1 and 2.2. Each kidney is surrounded by a thin fibrous capsule. On the medial aspect of the kidney is a vertical slit, the hilum, which leads to a central recess, the renal sinus (Fig. 2.2). The hilum transmits the renal vein anteriorly, the renal artery and, posteriorly, the pelvis of the ureter, together with renal nerves and lymphatics. Within the kidney the pelvis divides into 2 or 3 major calyces each dividing into a number of minor calyces. The renal parenchyma has an outer cortex containing glomeruli and convoluted tubules, and an inner medulla, containing loops of Henle and straight collecting duct. The medulla is disposed as a series of conical pyramids whose apices invaginate the minor calyces. The cortex extends as a series of projections between the pyramids (25-28).

The basic functional unit of the kidney is the nephron (Fig. 2.3). Each kidney has $1 – 1.5 \times 10^6$ nephrons. The nephrons have a complex and precise relationship to a rich blood supply. Blood vessels and nephrons are orientated to each other to facilitate secretion and reabsorption. The various segments of the nephron have different functions which are summarized in Table 2.1.

There are 3 major cell types within the glomerulus; endothelial cells which form the inner lining of the capillary, epithelial cells which line the inner aspect of Bowman's capsule, and mesangial cells which are distributed centrally between the capillary loops and at the vascular pole of the glomerulus (Fig. 2.4).

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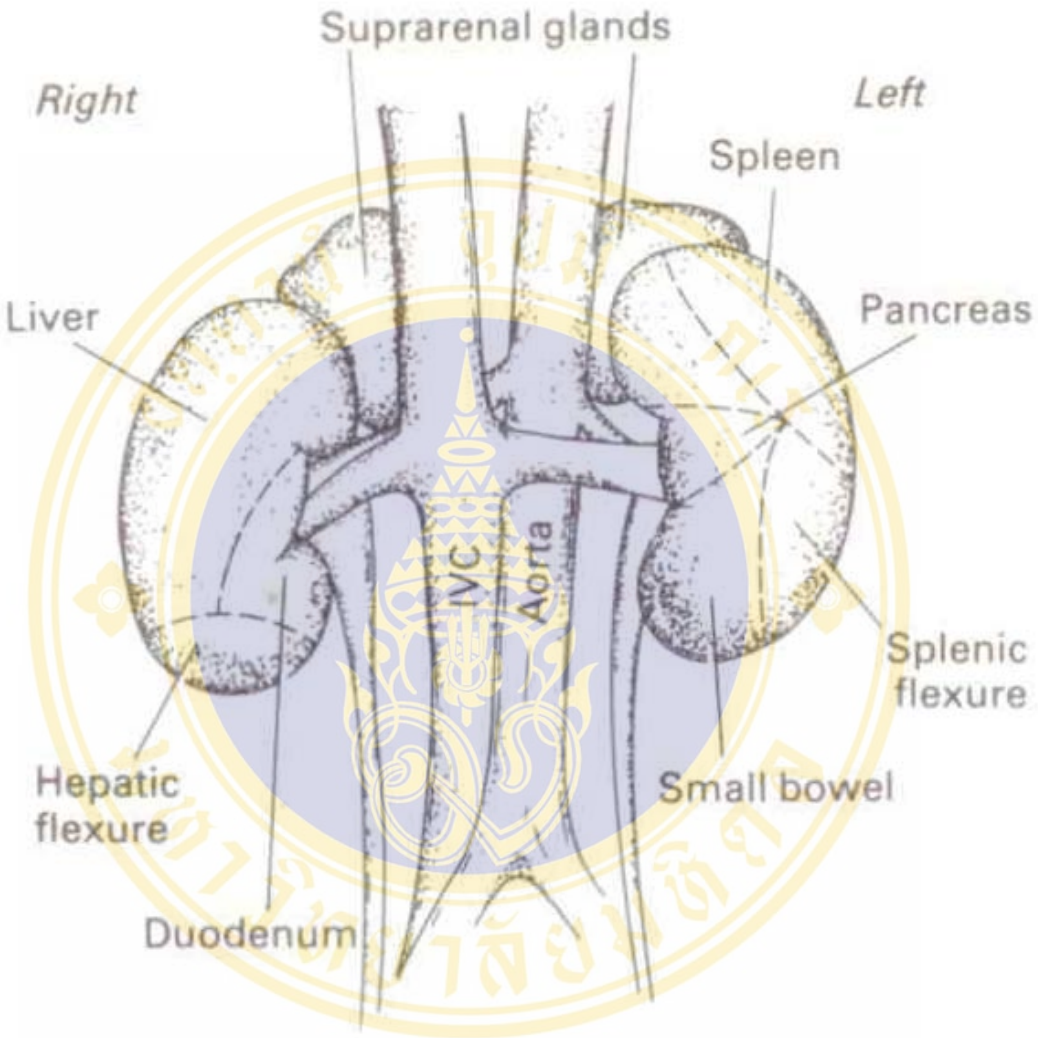

Figure 2.1 Anterior Relations of the Kidney (28).

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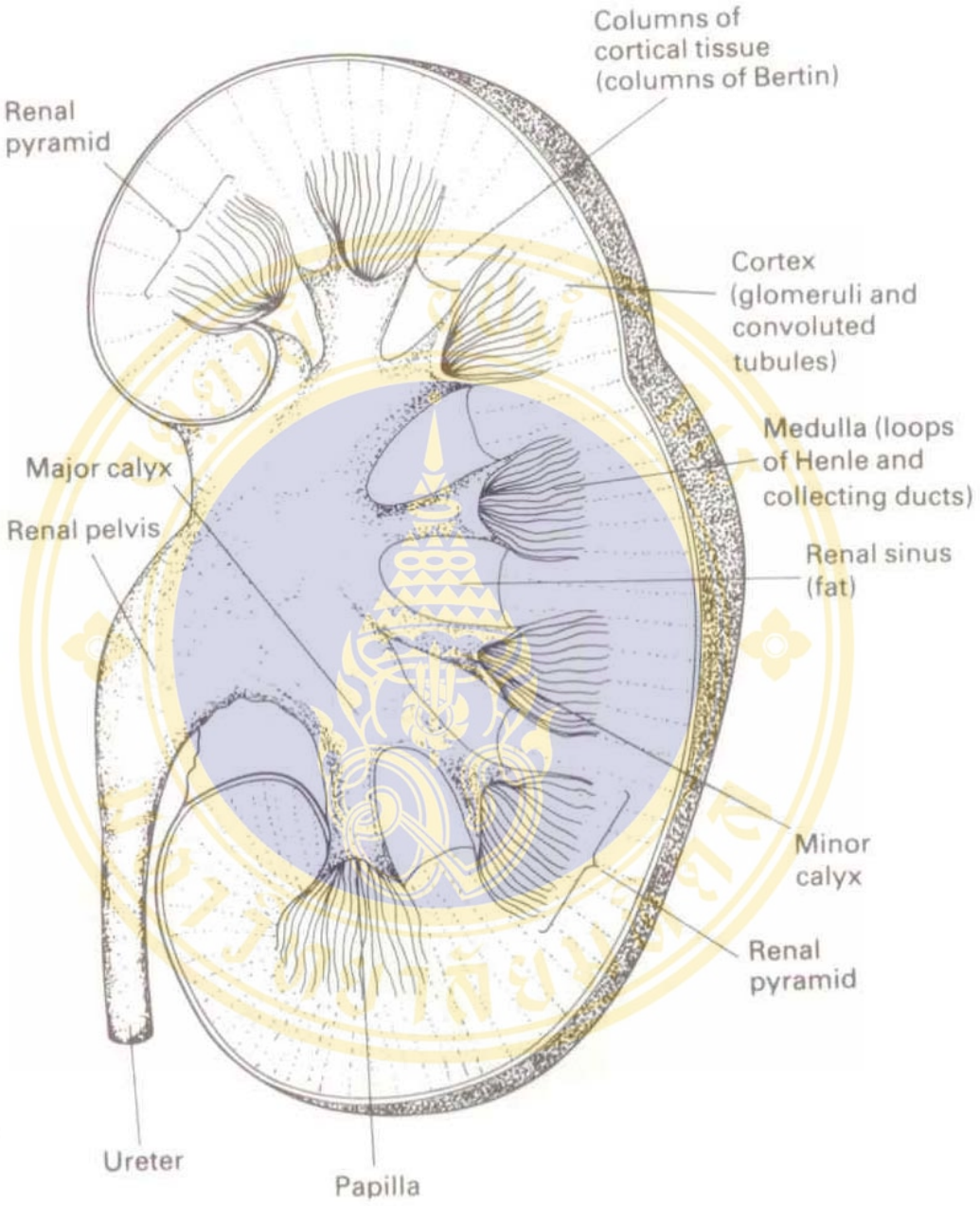

Figure 2.2 Macroscopic Appearance of the Kidney (28).

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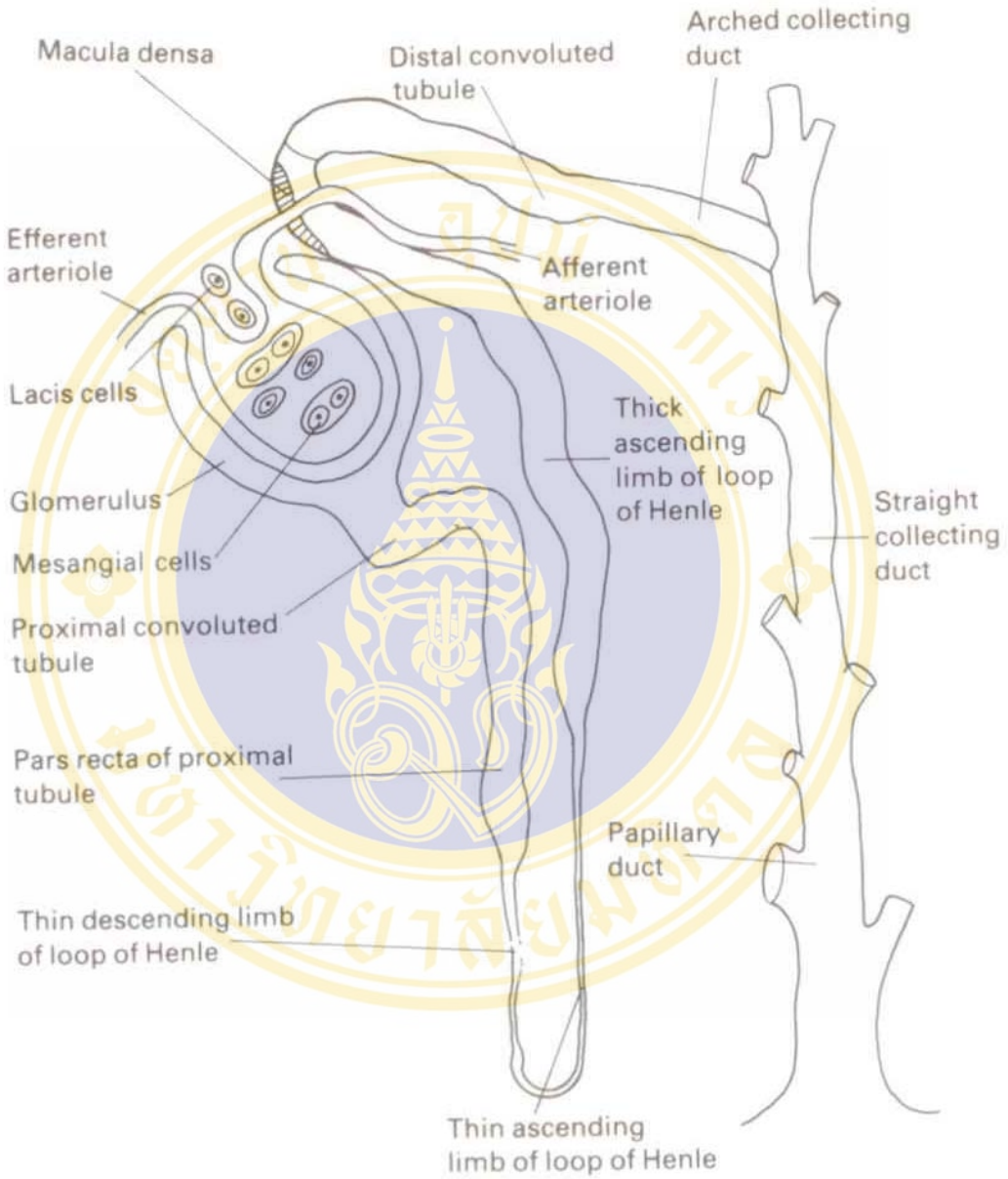

Figure 2.3 The Nephron; the Basic Functional Unit of Kidney (28).

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Table 2.1 Distribution of Function (28)

Segment	Function
Bowman's capsule Glomerulus Capillary tuft	Ultrafiltration
Proximal convoluted tubule	Bulk reclamation of sodium, chloride and water Excretion of Hydrogen ions Conservation: Glucose Amino acids Phosphate Bicarbonate Potassium Reabsorption of low molecular weight proteins
Loop of Henle Thin limp Thick limp	Conduit Sodium pump Excretion of free water (urinary dilution) Calcium reabsorption Magnesium reabsorption
Macula densa	Regulation of renin secretion
Distal convoluted tubule + Collecting ducts	Secretion: Hydrogen Potassium Ammonium Magnesium Reabsorption: Sodium chloride Magnesium
Collecting ducts	Water reabsorption and urine concentration (ADH) Sodium chloride reabsorption (aldosterone)

ADH = antidiuretic hormone.

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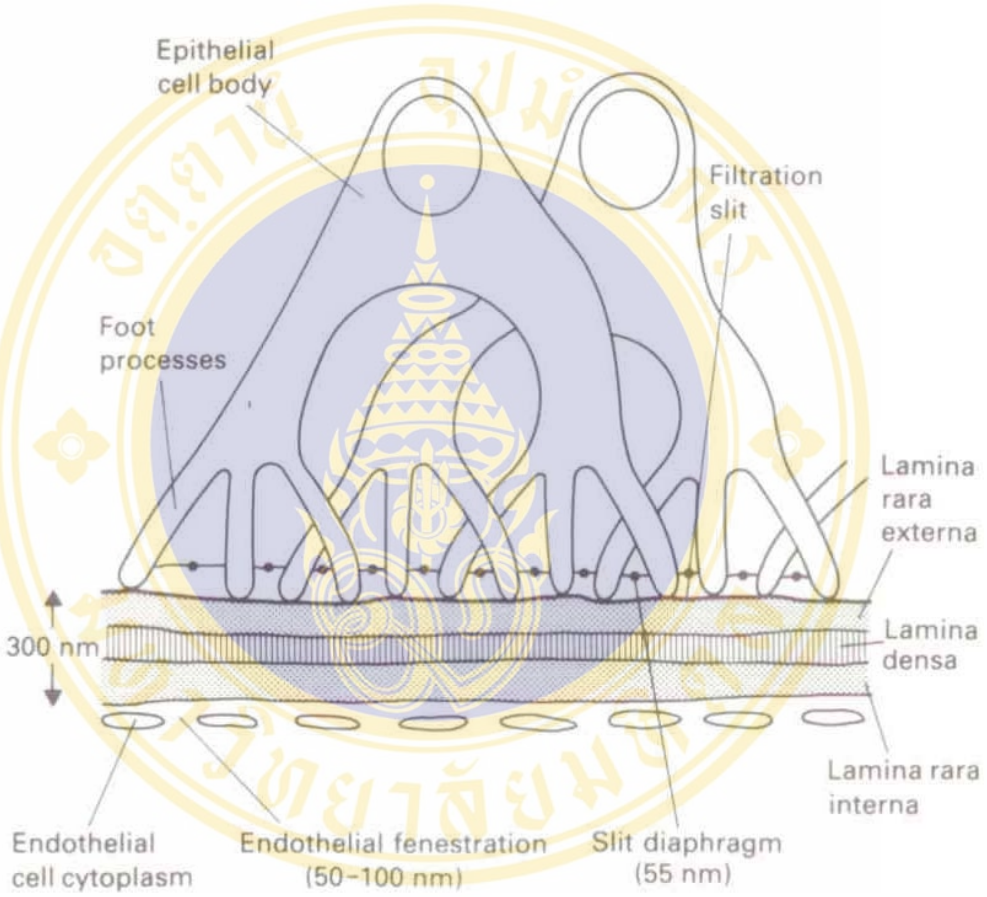

Figure 2.4 Glomerular Ultrastructure (28).

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The glomerulus acts as a high pressure filter which is capable of retaining large molecular weight substances according to size and electrical charge. The electrical charge is located principally on the epithelial cell foot processed. These negative charges retard the passage of negatively charged macromolecules across the filtration barrier. In disease in which there is loss of epithelial foot processes, there is also loss of this negative charge. Loss of foot processes is usually associated with heavy proteinuria (25-28).

2.2 Chronic Kidney Disease (CKD)

Kidney damage can happen quickly, but most often develops slowly and silently, often taking years or decades for the injury to become apparent. Gradual loss of kidney function is called “Chronic Kidney Disease”.

Chronic kidney disease includes conditions that damage kidneys and decrease their ability to keep health. If kidney disease gets worse, wastes can build to high levels in blood and make feel sick. The patients may develop complications like high blood pressure, anemia (low blood count), weak bones, poor nutritional health and nerve damage. Also, kidney disease increases a risk of having heart and blood vessel disease. These problems may happen slowly over a long period of time. Chronic kidney disease may be caused by diabetes, high blood pressure and other disorders. Early detection and treatment can often keep chronic kidney disease from getting worse. While kidney disease progresses, it may eventually lead to kidney failure, which requires dialysis or a kidney transplant to maintain life (1).

The two main causes of chronic kidney disease are diabetes and high blood pressure, which are responsible for up to two-thirds of the cases. Diabetes happens when blood sugar is too high, causing damage to many organs in the body, including the kidneys and heart, as well as blood vessels, nerves and eyes. High blood pressure, or hypertension, occurs when the pressure of blood against the walls of blood vessels increases. If uncontrolled, or poorly controlled, high blood pressure can be a leading cause of heart attacks, strokes and chronic kidney disease. Also, chronic kidney disease can cause high blood pressure (1).

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The National Kidney Foundation (NKF) has intended to set the stages of chronic kidney disease for help the physicians and patients have a good treatment plan.

The important factor to prevent the end-stage of kidney disease is early detection. When kidney disease is detected early, treatment such as dieting and taking medication can help to slow or prevent the progression of kidney diseases, toward kidney failure (29, 30). The National Kidney Foundation (NKF) suggests that the three simple tests can detect chronic kidney disease (1):

1. Blood pressure measurement. High blood pressure is the second leading cause of CKD. It also is a complication of CKD. The blood pressure target is less than 130/80 mg Hg.

2. Measurement of the level of creatinine (a waste that comes from normal muscle activity). When kidneys are damaged, creatinine can build to high levels in the blood. The results of creatinine blood test should be used to estimate glomerular filtration rate, or GFR. Your GFR tells how much kidney function has.

3. Measurement of protein in the urine. Protein is an important building block in the body, and kidneys normally reabsorb any filtered protein. When kidneys are damaged, however, protein leaks into the urine. Persistent protein in the urine indicates chronic kidney disease.

Many laboratories are searching for new markers that can detect the impaired kidney function in the early stages (31, 32).

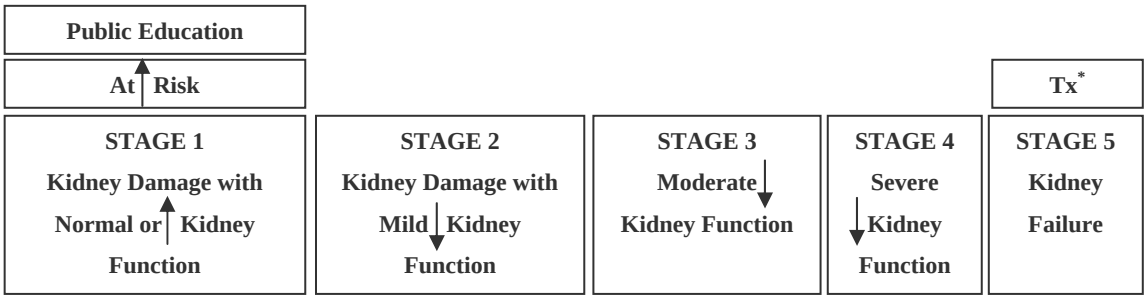

Figure 2.5 Stages of Chronic Kidney Disease (1)

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Table 2.2 Stages of Chronic Kidney Disease (1)

Stage	Description	Glomerular Filtration Rate (GFR)
At increased risk	**Risk factors** for kidney disease (e.g., diabetes, high blood pressure, family history, older age, ethnic group)	More than 90
1	Kidney damage (protein in the urine) and **normal** GFR	More than 90
2	Kidney damage and **mild** decrease in GFR	60 to 89
3	**Moderate** decrease in GFR	30 to 59
4	**Severe** decrease in GFR	15 to 29
5	**Kidney failure** (dialysis or kidney transplant needed)	Less than 15

2.3 Laboratory Evaluation of Kidney Function

2.3.1 Glomerular Filtration Rate (GFR)

The standard test used to measure or evaluate the filtering of the glomeruli, as kidney function, is the clearance test. Renal clearance has been defined as the volume of plasma cleared of a determined substance in its passage through the kidneys in a unit time. Homer Smith (33) was the first to propose that in order to be accepted as valid for the measurement of GFR, a substance must fulfill the following requirements, it must be metabolically inert and nontoxic, it must have no intrinsic effect on renal function and after being filtering freely at the glomerulus, and it must be neither reabsorbed nor secreted by the renal tubules. Furthermore, its clearance rate must not be affected by any other substance, it must remain constant regardless of variations in plasma levels and urine flow and it must be possible to measure its

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concentration in plasma and urine by simple, dependable, and accurate laboratory methods.

GFR can not be measured directly; it is assessed from renal clearance. The renal clearance of a substance *X* is given by the equation (34).

$$\text{Clearance of X (Cx)} = \frac{Ux \times V}{Px}$$

Where Cx is the clearance of *X* (ml/min)

Ux is the urinary concentration of X (mg/ml)

V is the urine floe rate (ml/min)

Px is the average plasma concentration of *X* during the period of urine collection

The clearance of inulin, a polysaccharide of molecular weight of approximately 5 kD, fulfills all these requirements and thus is the standard method for the measurement of GFR. However, inulin is not endogenous substance, it is required the intravenous administration. This procedure must be performed by specialized technical personnel; it requires that urine and multiple blood samples are collected at constant, well-timed intervals, and expensive in staff time. Because of these difficulties, alternative methods have been searched for the measurement of GFR.

The finding that creatinine is concentrated in the urine more than most of other endogenous substances that eliminated by the kidney (35). Creatinin is an amino-acid end product of protein metabolism that is derived from creatine. The main sites of creatine synthesis are the liver, pancreas, and the kidney. However, the bulk of creatine is contained in muscle, where creatine is stored as creatine and phospho-creatine before it is further metabolized and released into the circulation as creatinine, which is then excreted in the urine. Therefore, creatinine is in endogenous concentration in the body and does not need to be infused for evaluations of renal function.

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Several formulas have been developed to estimate creatinine clearance from serum creatinine concentration, age, sex and body size. The most widely used formula to predict creatinine clearance that proposed by Cockroft and Gault (36).

Creatinine Clearance (Ccr) = [(140-age) × weight] / (Serum Creatinine × 72) for men
= [(140-age) × weight] / (Serum Creatinine × 85) for women

This formula is used to detect the onset of renal disease, and to evaluate the effectiveness of therapy for kidney disease. The National Kidney Foundation recommended evaluating the GFR from the calculation of serum creatinine (1). However, the errors of this prediction were 21 %– 27 %, which is not less than the standard deviation of the range of Ccr at a given age and surprisingly (37).

2.3.2 Blood Urea Nitrogen (BUN)

Urea is the end product of protein catabolism and its synthesized primary by the liver. About a quarter of synthesized urea is metabolized in the intestine to carbon dioxide and ammonia and the ammonia thus generated return to liver where it is reconverted to urea. Urea is usually measured as urea nitrogen; the serum level is commonly referred to as the blood urea nitrogen (BUN). Urea is not only filtered by the glomeruli but also partially reabsorbed by tubules. Degeneration of kidney function leads to a predictable increase in BUN. When 50 % of kidney function is reduced it results in doubling the BUN in the steady state. BUN, that widely used to assess kidney function, is less satisfactory measurement than serum creatinine chiefly because of two factors. The first, urea clearance varies with the rate of urine flow and secondly, the plasma concentration of urea is importantly depend on nitrogen metabolism as well as renal function (4, 5).

2.3.3 Blood test of Creatinine

Creatinine is the waste product produced in your muscles from the breakdown of a compound called creatine. Creatine is the part of the cycle that produces energy needed to contract the muscle and it as well as creatinine are produced at a relatively constant rate. Almost all creatinine is excreted by the kidneys, so blood levels are a

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good measure of how well in kidney function. The quantity produced depends on the size of the person and their muscle mass. For this reason, creatinine concentrations will be slightly higher in men than in women and children. Serum creatinine is roughly, inversely related to clearance test. If the clearance drops to one half of the old level, the serum creatinine doubles (in the steady state) (38, 39). Unlike the BUN, serum creatinine is not affected by protein metabolism and by the patient's hydration status, only kidney function changes that make the results of serum creatinine (40). Unfortunately, the serum creatinine is an insensitive marker of kidney damage that is serum creatinine does not rise until at least half of the kidney's nephrons are destroyed. Thus, estimates of GFR based on serum creatinine alone are incapable of detecting a glomerular injury until overpower impairment of the glomerular capillary wall function has occurred (6, 7, 39, 40).

2.3.4 Urinalysis

A urinalysis is a simple, inexpensive test that can help to detect problems in many parts of the body including kidneys and urinary tract, heart and liver. A urinalysis can help to detect many diseases before symptoms occur. Early detection and treatment can often prevent serious diseases from getting worse. A urinalysis has three parts (1):

2.3.4.1 Visual examination of the urine sample for color and clearness. Blood may make urine look red or the color of tea or cola. An infection may make urine look cloudy.

2.3.4.2 A dipstick examination, which uses a chemically treated strip to check for the following:

2.3.4.2.1 pH is a measure of the amount of acid in the urine. An abnormal pH may be a sign of kidney stones, urinary infections, chronic kidney disease or certain disorders that affect growth and development in children.

2.3.4.2.2 Protein is an important building block in the body. When kidneys are damaged, protein leaks into urine. Persistent protein in protein in the urine suggests that the kidney's filtering units have been damaged by chronic kidney disease.

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2.3.4.2.3 Urine Creatinine gives an estimate of the concentration of urine, which allows for a more accurate protein result. Creatinine is a by-product of normal muscle activity, which is found in the urine and blood.

2.3.4.2.4 Glucose (sugar) is usually a sign of diabetes. In children, sugar in the urine may sometimes be related to a disorder that affects growth and development.

2.3.4.2.5 Bacteria and white blood cells (pus cells) are signs of infection. Bacteria without white blood cells may suggest another type of problem such as vaginal or bladder disease.

2.3.4.2.6 Bilirubin is a waste product from the breakdown of old red blood cells. It is normally removed from the blood by the liver and becomes part of bile. Its presence in the urine may be a sign of a liver disease.

2.3.4.3 Urinalysis also includes examining a small amount of urine under a microscope. Some of the things that may be seen include:

2.3.4.3.1 Red blood cells, which may be a sign of kidney diseases that damage the filtering units of the kidneys, allowing blood cells to leak into the urine. Blood in the urine may also be a sign of problems like kidney stones, infections, bladder cancer or a blood disorder like sickle cell disease. While enough blood may be present to cause a color change that can see, blood in the urine is usually so small that a microscope is needed to see it.

2.3.4.3.2 White blood cells (or pus cells), which are a sign of an infection or inflammation in the kidneys, bladder or another area.

2.3.4.3.3 Bacteria, or germs, which are usually a sign of an infection in the body.

2.3.4.3.4 Casts, which are tube-shaped forms made of protein, and may have red or white blood cells or other cells inside. Casts form in certain kidney diseases because the kidneys are releasing a sticky type of protein that traps blood cells and other types of cells.

2.3.4.3.5 Crystals, which are formed from chemicals in the urine. If they become large enough, they form kidney stones.

The urinalysis of protein, laboratory analysis is still necessary for the differentiation of abnormal from normal protein output and the assessment of kidney

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disease. There is evidence to suggest that a proceeding phase of albuminuria, characterized by concentrations of albumin above normal but not detectable by conventional dipstick tests may predict clinical proteinuria.

2.4 Proteinuria

Proteinuria (from *protein* and *urine*) means the presence of an excess of serum proteins in the urine. Proteinuria may be a sign of kidney damage. Since serum proteins are readily reabsorbed from urine, the presence of excess protein indicates either an insufficiency of absorption or impaired filtration. Diabetics may suffer from damaged nephrons and develop proteinuria.The abnormal passage of plasma protein into the urine is the fundamental process ultimately responsible for all of the clinical and biochemical changes that characterize the nephrotic syndrome. The protein that is excreted in urine under physiological conditions is not usually detected on urinalysis or dipstick testing. Pathological proteinuria has been classified into four groups (1, 41-44):

- Orthostatic proteinuria is a urine protein excretion increases with activity and upright posture, and so the protein content of urine passed late in the day will always be greater than that passed first thing in the morning. This is not usually detectable on dipstick testing but the physiological increase is overstated occasionally. Orthostatic proteinuria occurs in some older children and teenagers. The word orthostatic means "upright." The condition is called "orthostatic proteinuria" because protein goes into the urine only when the child is standing up. Patients with this condition have no kidney damage but, for some unknown reason, they lose protein into the urine during the day when they are active. At night, while they sleep, their kidneys don't let any protein into the urine. The doctor can diagnose this harmless condition by checking 2 urine samples. The first is collected in the morning; right after patients gets up. The second sample is collected throughout the day. The samples are kept in separate containers. If patients have orthostatic proteinuria, the morning sample won't have protein in it, but the urine collected during the day will have protein in it. If orthostatic

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proteinuria is confirmed and there is no significant proteinuria on a recumbent sample, the patients may be reassured and no follow-up is necessary.

- Glomerular proteinuria occurs because of increased glomerular permeability to proteins. Glomerular proteinuria occurs when there is injury to the glomerular capillary wall by processes such as immune complex deposition (e.g., systemic lupus erythematosus), accumulation of abnormal glomerular basement membrane (e.g., diabetes), or injury to the glomerular podocyte (e.g., minimal change disease, also called minimal change nephrotic syndrome). These disruptions increase permeability of the glomerular capillary by increasing glomerular pore size or decreasing electro-negativity of the capillary wall (which normally repels albumin), or both. Microalbuminuria that is the result of endothelial dysfunction is also an example of glomerular proteinuria. However, in contrast with the mechanisms already described here, endothelial dysfunction alone is less likely to lead to progressive loss of kidney function (45-47).

- Tubular proteinuria is due to decreased tubular reabsorption of proteins contained in glomerular filtrate and is seen in tubulo-interstitial diseases. Normally, the glomerulus filters low-molecular-weight proteins (e.g., $beta_2$-microglobulins) that are nearly completely reabsorbed by the tubules. In patients with tubular injury (e.g., chronic pyelonephritis, toxin-induced or drug-induced interstitial nephritis, Fanconi's syndrome), these proteins are excreted in urine. Albuminuria is minimal because glomerular permeability is generally intact in these patients

- Overload proteinuria low-molecular-weight proteins overwhelm the ability of the proximal tubules to reabsorb filtered proteins. Most often, this is a result of the immunoglobulin overproduction that occurs in multiple myeloma. The resultant light-chain immunoglobulin fragments (Bence Jones proteins) produce a monoclonal spike in the urine electrophoretic pattern. The myeloproliferative conditions that cause this form of proteinuria are rare in children.

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2.5 Microalbumin

Albumin is the most abundant protein in the blood plasma. It keeps fluid from leaking out of blood vessels; nourishes tissues; and transports hormones, vitamins, drugs, and ions like calcium throughout the body. Albumin is made in the liver and is extremely sensitive to liver damage. The concentration of albumin drops when the liver is damaged, with kidney disease, when a person is malnourished, if a person experiences inflammation in the body, or with shock. Albumin increases when a person is dehydrated.

A microalbumin test evaluates urine for the presence of a protein called albumin. Albumin is normally found in the blood and filtered by the kidneys. When the kidneys are working properly, albumin is not present in the urine. However, when the kidneys are damaged, small amounts of albumin leak into the urine. This condition is called “Microalbuminuria”; the term ‘micro-’ refers to low concentrations of urinary albumin.

Mogensen and Schmitz defined the presence of microalbuminuria as ‘the incipient stage of nephropathy’ and related it to early glomerular hyperfiltration (48). The American Diabetes Association (ADA) recommends that people with diabetes who past puberty and have diabetes for more than five years have the amount of protein in their urine measured at least once each year (11). The National Kidney Foundation (NKF) recommends three simple tests can detect chronic kidney disease: blood pressure, serum creatinine, and urine albumin (1).

The pathophysiology underlying microalbuminuria is believed to include two major classes of factors: elevations in intraglomerular pressure, and structural or functional changes of the mesangium and the glomerular basement membrane. Increased intraglomerular pressure may be caused by an imbalance in afferent and efferent renal vascular tone, increased blood flow and extracellular fluid volume. Together, elevated hydrostatic and oncotic pressures initially cause renal hyperfiltration. Mesangial hypertrophy and proliferation, as well as changes in the polyanionic charge and porosity of the glomerular basement membrane in the kidney and in other vascular sites (e.g., retina, arteries, nerve), are caused by deposition of advanced glycosylation end products (as in diabetes) and perhaps other, as yet unidentified substances. In addition, deposition of lipids (as in atherosclerosis) and

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immune complexes (as in inflammatory conditions) may contribute to altered porosity of vascular basement membranes. Negatively charged albumin molecules normally have little glomerular permeability because they are repelled by the negatively charged membrane surface. However, changes in basement membrane polyanionic charge accompanying a variety of metabolic and immune disorders results in increased porosity and loss of microvascular integrity, resulting in leakage of albumin and other substances into the renal tubule and pericapillary tissues of nerves, arteries and retina.

A variety of analytical methods is available for measuring urinary albumin, but a highly accurate quantitative immunoassay specific for albumin is ideal. The first trial for measurement of low albumin concentrations in urine was made using radioimmunoassay (RIA) (49, 50). Although RIA is still generally considered to reference method for urinary albumin but the radio-labeling have certain disadvantages, it is expensive, it has a relatively short shelf life and its use is possible only in laboratories to allow handling radioactive substances. Enzyme and fluoroimmunoassay have more stable reagents but these methods are expensive, needed labeling substances, and time consuming. Immunonephelometry (INM) and immunoturbidimetry, a newer technique with full automation that has became the major method for protein quantification, requires artificial facilities and fixed matched reagents and offers suboptimal precision, all of which limit its clinical applications (19-21, 49, 51, 52). Moreover, a enzyme-link immunosorbent assay (ELISA) (53, 54), a chemiluminescence immunoassay (55), a latex particle counting immunoassay (56), a colorimetric liposomal immunoassay (57), high-performance liquid chromatography (HPLC) (49) was developed for microalbumin determination but its have many disadvantage. Therefore a fast, sensitive, label-free, and easy-to-manipulate technique for urinary protein quantification is needed for clinical use.

2.6 Biosensor

The biosensor technology began in several decades ago. Research trends nowadays are suggested newly developing chemical and biological sensors, which are simple and inexpensive to design. Biosensors are generally regarded as analytical devices comprising a biological substance connected to a suitable physical transducer

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which converts the biochemical response into electrical signal (Fig. 2.6). There are many types of biological substances that can be used for biosensors include enzymes, oligonucleotide, organelles, membranes, cells, antibodies, affinity ligand, receptors and peptide. The importance of the biological substance is that its interaction with the analyte is highly specific to that analyte alone. When biological molecules interact with target analytes, there is a change in one or more physical-chemical parameters associated with the interaction. The changing may generate ions, gases, electrons, heat, mass or light. The suitable transducer converted these quantities into electrical signals, which are amplified and displayed in a suitable device (58).

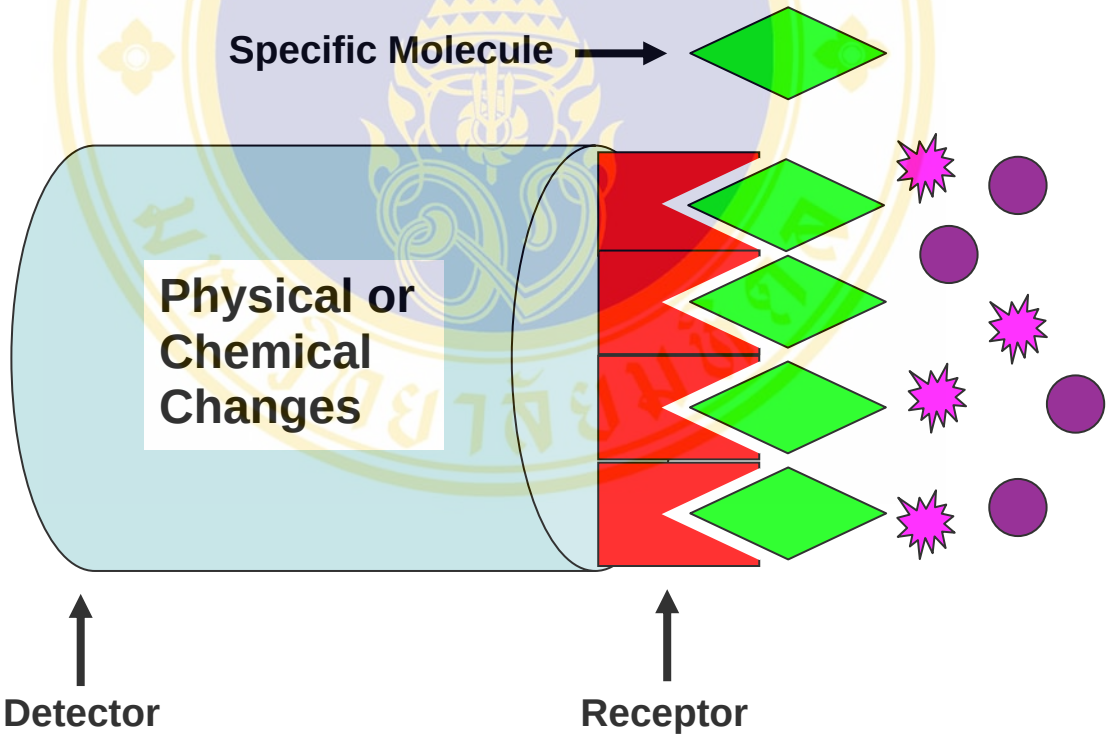

Figure 2.6 Schematic Diagram of Biosensor

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From these features, the specificity of biosensor depends on the properties of the biological component which function is to provide selectivity and specificity for target analyses. Whereas, the sensitivity depends on both the biological component and transducer due to significant of reaction between biological substances and target analyses and a high efficiency of transducer (59). There are two different types of biosensors: biocatalytic and bioaffinity-based biosensors. The biocatalytic biosensor uses mainly enzymes as the biological compound, catalyzing a signaling biochemical reaction. The bioaffinity-based biosensor, designed to monitor the binding event itself, uses specific binding proteins, receptors, nucleic acids, membranes, whole cells, antibodies or antibody- related substances for biomolecular recognition. In the latter two cases, these biosensors are called immunosensors.

2.7 Immunosensor

The type of biosensor has been named upon the type of biological molecules. Immunosensors are defined as analytical devices that detect the binding of an antigen to its specific antibody by coupling the immunochemical reaction to the surface of a device known as a transducer (Fig. 2.7). They have been the subject of increasing interest during the past decade mainly because of their potential application as an alternative immunoassay technique in areas such as clinical diagnostics and environmental control. Regardless of the transducer or application involved, an ideal system would have the following specifications: first, an ability to detect and quantify the antigen, within the required concentration range and a reasonable time (preferably a few seconds); second, a capacity to transduce the binding event without externally added reagents; third, a capacity to repeat the measurement on the same device (i.e. the immunochemical reaction should be reversible); and fourth, the ability to detect the specific binding of the antigen in real samples (60-62).

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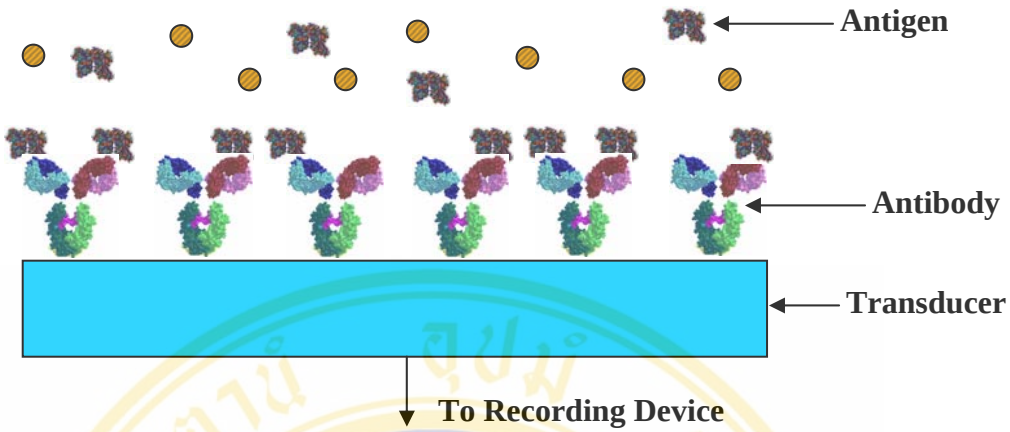

Figure 2.7 Schematic Diagram of Immunosensor

Today, very few systems, if any, possess all of the above properties. Nevertheless, neither the commercial potential nor the importance of immunosensors as an alternative immunoassay technique should be underestimated. The increasing amount of immunosensor-related research published every year, as well as the continuous development of both physical and biochemical aspects of immunosensors indicate the enormous significance still attached to this research field. Currently, immunosensors offer scientists a powerful tool for monitoring biospecific interactions in real time and for deriving information about the binding kinetics of the immunoreactions or the structure of molecules and their biological function (63-65).

Depending on the transducer technology employed, immunosensors can be divided into three principal classes: optical, piezoelectric and electrochemical. Furthermore, on the basis of the immunoassay format used, they can be either direct (where the immunochemical reaction is directly determined by measuring the physical changes induced by the formation of the complex) or indirect (where a sensitively detectable label is combined with the antibody or antigen of interest). A sufficiently high selectivity may be obtained with non-labeled immunosensors, although non-specific adsorption onto the biorecognition surface remains a major problem for direct systems (60, 61).

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2.8 Piezoelectric Immunosensors

Recent advances in transducer technology have stimulated great interest in piezoelectric devices. The utility of the piezoelectric device as a mass sensor arises from the linear relationship between the change in the mass at the crystal surface and the change in its oscillating frequency (62). The vibration of piezoelectric crystals produces an oscillating electric field in which the resonant frequency of the crystal depends on parameters associated with the phases adjacent to the crystal and on the physical properties of the crystal itself (e.g. size, cut density and shears modulus). The change in mass is identified by a corresponding change in the frequency of vibration of the crystal. The fundamental frequency increases as the piezoelectric thickness decreases. The change in frequency (ΔF) resulting from any added mass (Δm) to the device can be described by the Sauerbrey equation (66).

$$\Delta F = \frac{-2 f_0^2 \Delta m}{A\sqrt{\mu_q \rho_q}}$$

Where ΔF= measured frequency shift, in Hz; f_0 = the fundamental resonant frequency of the crystal, in Hz; Δm = mass change, in g; A = piezoelectrically active area (area of electrode surface), in cm^2; μ_q = shear modulus of quartz = 2.947 x 10^{11} g/cm^2; ρ_q = density of quartz, 2.648 g/cm^3.

There are two main types of piezoelectric device: first, the quartz crystal microbalance (QCM), which operates at frequencies below 15 MHz; and second, the surface acoustic wave (SAW) device, which operates at frequencies normally above 100 MHz.

A SAW crystal oscillate at several hundred megahertz, instead of 9-14 MHz for the piezoelectric crystal. In this technique an acoustic wave is generated by application of an alternating voltage across a pattern of interlaced metal electrode (e.g. gold). These devices are more mass sensitive than piezoelectric quartz crystal because mass sensitivity is directly related to the operating frequency (67).

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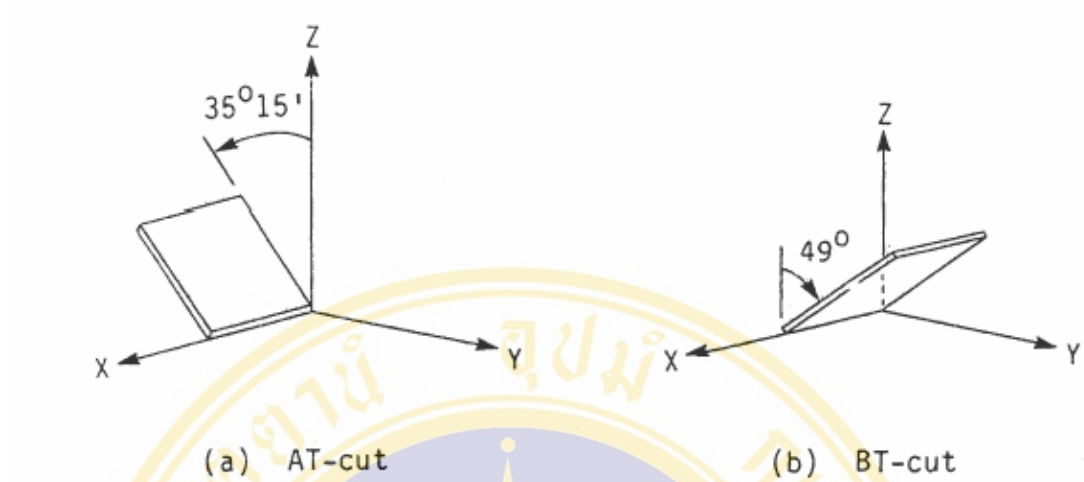

Figure 2.8 AT- and BT- Quartz Crystals (67)

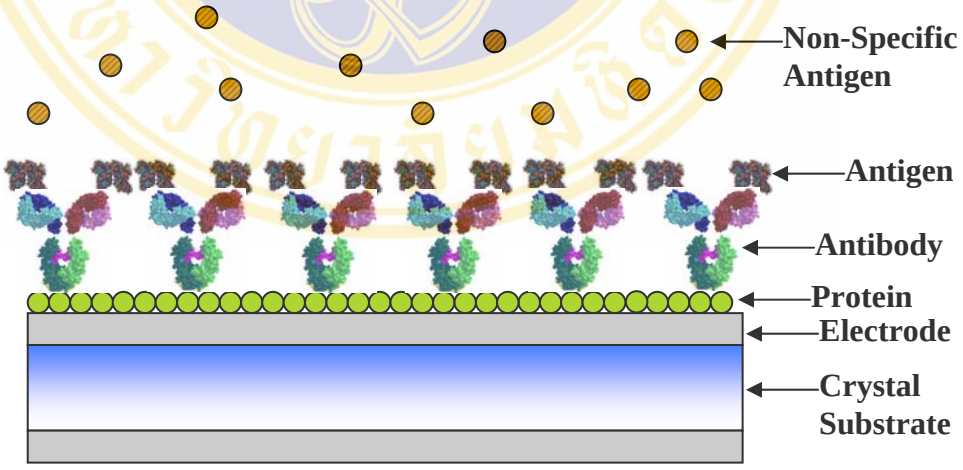

Figure 2.9 Schematic Diagram of Ab-Ag Complex on QCM

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A piezoelectric quartz crystal resonator is a precisely cut slab from a natural or synthetic crystal of the quartz. The quartz crystal plate must be cut in a specific orientation with respect to the crystal axes. These cuts belonging to the rotated Y-cut family, the AT- and BT-cuts shown in Figure 2.8 are representative. For the majority of piezoelectric work in analytical, AT-cut crystal have been used. The advantage with the AT-cut quartz crystal is that it has nearly zero frequency drift with temperature around room temperature (67). The AT–cut refers to quartz wafers cut at $+35^0$ 15′ angle from the Z axis. Other terms used to references or describe this same piezoelectric quartz crystal are: quartz crystal microbalance (QCM) and thickness shear mode (TSM). The AT-cut quartz crystals as piezoelectric material can function in a "microbalance" mode as quartz crystal microbalance. TSM is used because it describes the motion of the crystal's vibration (68). A quartz crystal is sandwich between two electrodes, which are generally composed of gold or silver and are prepared by thermal evaporation on to the quartz surface (69).

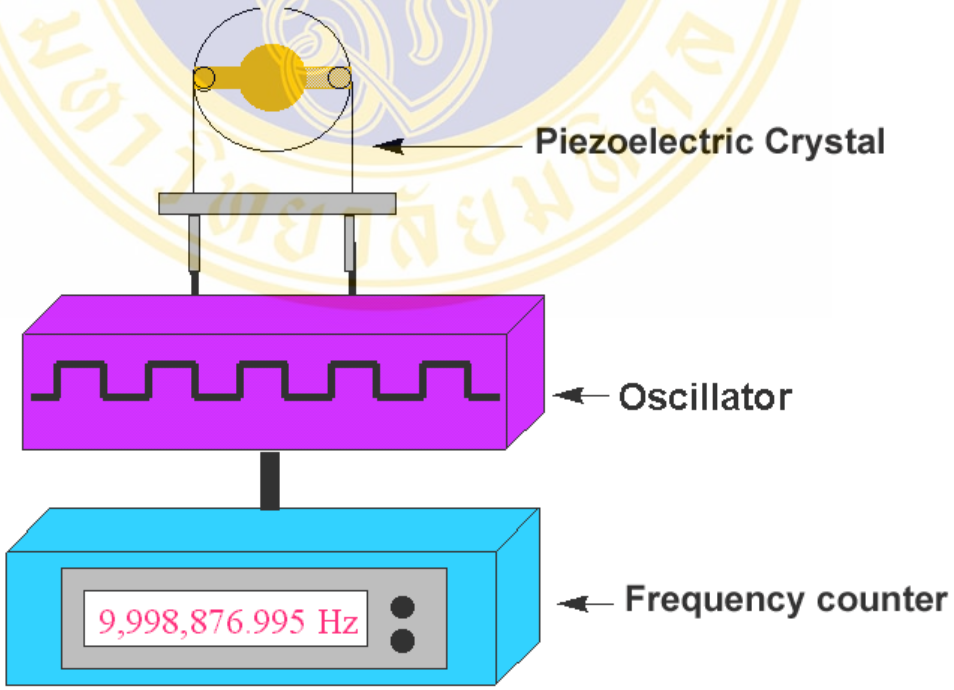

Figure 2.10 Experimental Apparatus of QCM

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The considerable number of papers that have appeared on piezoelectric immunosensors indicates the potential of these systems as low-cost sensors for one-step detection of immunochemical reactions (Fig. 2.9). Developments in piezoelectric immunosensors and their applications, including the monitoring of antigen binding, cell binding or virus binding to the device surface, have been reviewed recently (70).

Actually, the principle limitation of the quartz crystal microbalance is non-specific adsorption of molecules present in real matrices. QCM is a mass sensor surface and any molecule able to bind to the surface is a potential in interferent. Method to improve the sensitivity of piezoelectric biosensor can be improved by using crystals of higher frequencies, but these devices are often difficult to operate in liquids because of fragility and frequency stability problems (71). Alternatively, the selectivity can be improved by improving the quality of the surface of the crystal and the immobilization procedure.

2.9 Antibody Immobilization

Immobilization is the process of attaching biological components to the transducer which can be separated into five methods (72).

1) **Encapsulation** uses biomaterial helping in place behind a membrane for closely contacting between the biomaterial and the transducer. This technique has many advantages; to be closely attachment between the biomaterial and the transducer, to be very adaptable, and to be very reliable specific to biomaterial (e.g. high specificity and good stability to change in temperature, pH, ionic strength, and substrate concentration. Some examples of encapsulations are polycarbonate and Teflon.

2) **Entrapment** is the technique by mixing between biomaterial and monomer solution, then mixer solution is polymerized to a gel, trapping the biomaterial. The most used gels are polyacrylamide, starch gels, nylon, silastic gels, and polypyrole. Unfortunately, this method can cause barrier to the diffusion of substrate, thus slowing the reaction and then slow response time of biosensor.

3) **Cross-linking** is the attachment, which is chemical bond to solid supports or to another support such as gels e.g. glutaraldehyde. This method stabilizes absorbed to

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biomaterials, but has limits diffusion and may be damage biomaterial and mechanical strength is poor.

4) **Covalent bonding** consist of some function groups, such as -SH, -OH, -CO_2H, -C_6H_4OH, and -NH_2, which can be covalently bonded to the support matrix (transducer or membrane). This bonding type is suitable during using because enzyme or biomolecules will not release and protect the actives site of biomolecules. Nevertheless, this type of bond should be under mild condition.

5) **Adsorption** is the simplest and minimal preparation, but molecule attachment is weak. The absorption is a weak bond via the formation of Van der Waals, occasionally with hydrogen bonds or change-transfer forces. The adsorption method has sense to change in pH, temperature, ionic strength and the substrate.

Every method can be amounted to change the structure of the electrode material at the surface or to coating the surface with a new material which have a highly specificity, sensitivity, and reproducibility of biosensor.

For the antibody immobilization technique in immunosensor, antibodies have to be properly immobilized on the immunosensor surface, which is the reaction between antibodies and analyses occurs. The optimum density and adjusted orientation of the antibodies are of paramount importance. There are four different types of oriented coupling of antibodies (73): binding to Fc receptors such as protein A or G on the surface; binding of other binding partners to structures, covalently linked to the Fc part of the antibody, e.g., the biotin residue on the Fc binds to surface-coated streptavidin; coupling to the solid support via an oxidized carbohydrate moiety on the CH-2 Fc domain; and the binding of Fab or scFv fragments to the surface of the device via a sulfhydryl group in its C-terminal region.

In this study, it is emphasis at adsorption technique because it is suitable for initial study, inexpensive, and easily to work. Physical adsorption is relatively simple and regularly used for antibody and receptors. The most one of this method is using Protein A, is produce from *Staphylococcus aureus*, gram positive bacteria. Protein A binds to F_c part of antibodies with specifically, therefore F_{ab} region of antibodies properly orient bind to epitope of antigen.

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CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

Chemicals used in this study were analytical and molecular biological grades.

- Bovine serum albumin (BSA), Sigma, USA.
- Human albumin, Sigma, USA.
- Hydrochloric acid (HCl), Merck, Germany.
- Polyclonal anti-human albumin, Sigma, USA.
- Potassium chloride (KCl), Sigma, USA.
- Potassium phosphate (KH_2PO_4), Sigma, USA.
- Protein A, Sigma, USA.
- Sodium chloride (NaCl), Sigma, USA.
- Sodium phosphate dibasic heptahydrate, ($Na_2HPO_4.7H_2O$), Sigma, USA.
- Tina-quant® Albumin, Roche Diagnostic company, Germany.

3.1.2 Equipments and Apparatus

- An AT-cut piezoelectric quartz wafer 12 MHz with silver electrode, 4 mm X 4 mm X 100 Angstroms, Kyocera Kinseki, Thailand (Fig. 3.1).
- Analytical balance, Model XT 220A, Precisa Instruments AG, Switzerland.
- Autoclave, Model Hiclave HV-85, Hirayama, Japan.
- Automatic pipettes; 20-1000 µl, Eppendrof Inc., Hamburg, Germany
- Desiccators, Nikkon, Japan.

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- Fume Hood, Staftech CO., LTD, Thailand.
- Hitachi 917, Automatic Analyzer Boechringer Mannheim, Roche Diagnostic company, Germany.
- pH meter, Model MP220, Mettler Toledo, Switzerland.
- Precisa XT 2200C, Precisa Instruments LTD., Switzerland.
- UV-VIS Spectrophotometer, Model CE 2040, Cecil, England.
- Oscillation Counting Device, In-house development (74) (Fig. 3.2).

3.1.3 Urine Samples

The study was performed in the Faculty of Medical Technology, Mahidol University and approved by the Committee on Human Rights Related to Human Experimentation, Mahidol University (No. MU 2007-066). A morning urine samples were collected from 64 people who come for health check-ups at Center of Medical Laboratory Service and completely examined the protein value by strip test. Thirty-two urines with negative result of protein by strip test and 32 urines with positive result of protein by strip test were selected and kept into an eppendrof tube without additives or preservatives, and placed into a cool box. After transferring to the laboratory, they kept in the refrigerator at -20^{o}C, with no freeze-thaw cycles before the protein assay. The microalbumin concentration of each specimen was tested on the day of collection or within 1 week. Each sample was further divided into 2 portions before detection. One portion was measured with immunoturbidimetry (conventional method) and the other with mass sensitive immunosnsor (proposed method).

3.2 Reagents Preparation

3.2.1 10x Phosphate Buffer Saline (PBS), pH 7.4

The solution of 10x PBS pH 7.4 was prepared by dissolving 80 g of Sodium Chloride (NaCl), 11.5 g of Sodium Phosphate, Dibasic, Heptahydrate ($NaHPO_4.7H_2O$), 2 g of Potassium Chloride (KCl), and 2 g of Potassium Phosphate (KH_2PO_4) in 800 ml of distilled water. The pH of PBS buffer was adjusted to 7.4 with 6N Hydrochloric acid (HCl). The total volume of the solution was adjusted to 1 L with

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distilled water. This solution was also autoclaved at 121 ^{o}C, 15 psi for 30 min and then kept at room temperature.

3.2.2 1x Phosphate Buffer Saline (PBS), pH 7.4

One volume of 10xPBS was added to 9 volumes of distilled water and then autoclaved at 121 ^{o}C, 15 psi for 30 min. This solution was kept at room temperature.

3.2.3 1% Bovine Serum Albumin (BSA) Solution

One mg of Bovine Serum Albumin powder was dissolved in 100 ml of distilled water. This solution could be kept at 4 ^{o}C for one week.

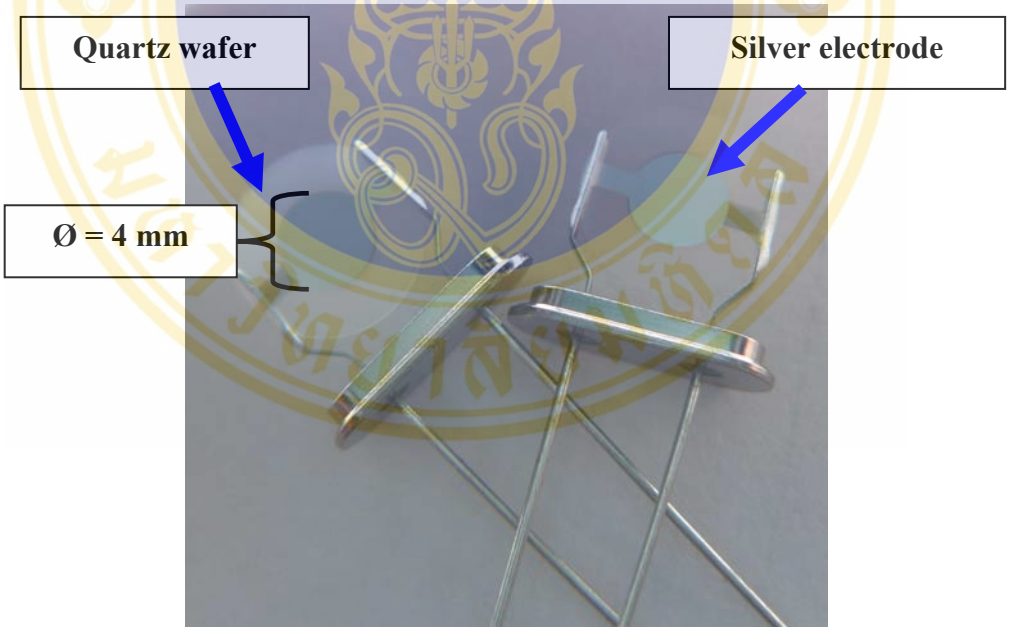

Figure 3.1 Schematic of 12 MHz AT-Cut Piezoelectric Quartz Wafer with Silver Electrode

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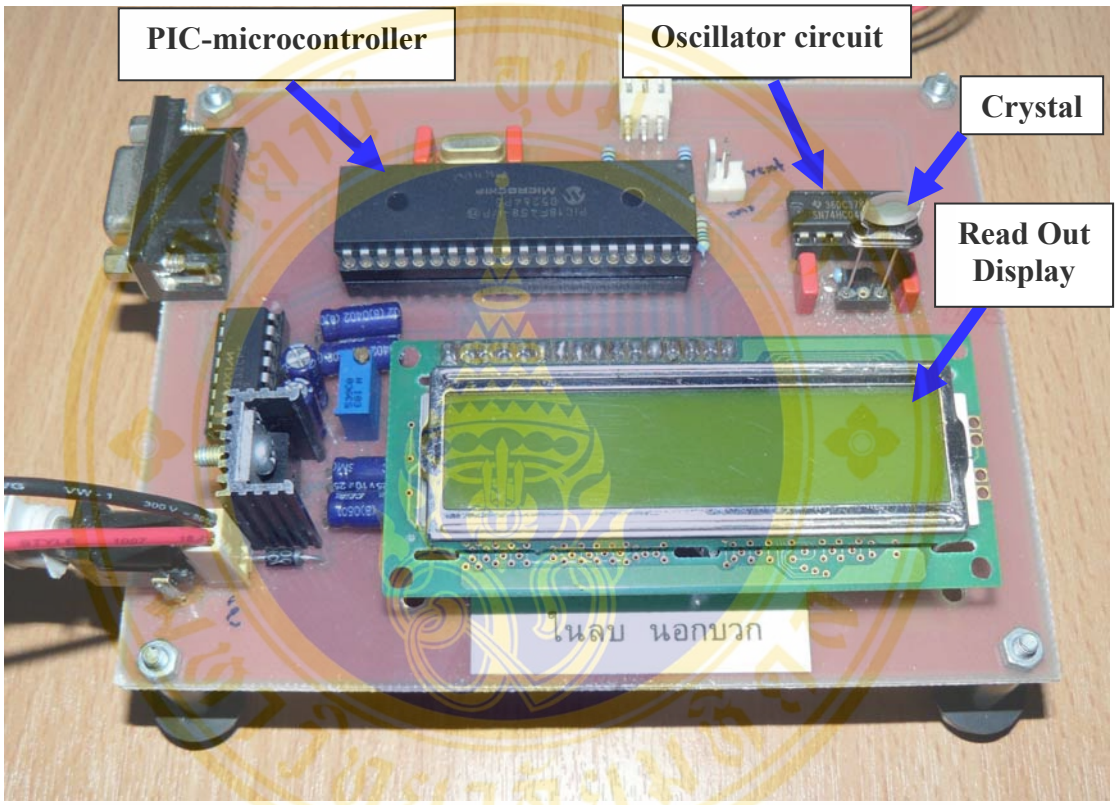

Figure 3.2 Oscillation Counting Device (74)

This device used for measuring the frequency change of the quartz crystal due to the addition of immobilization material. The major composition of this device composes of PIC-microcontroller, oscillator circuit and read out display.

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3.3 Methods

3.3.1 Calculation

The change in frequency (ΔF) resulting from any added mass (Δm) to the device can be described by the Sauerbrey equation (66).

$$\Delta F = \frac{-2 f_0^2 \Delta m}{A\sqrt{\mu_q \rho_q}}$$

Where ΔF is the change in frequency (Hz); f_0 is the resonant frequency of the crystal (MHz); Δm is the mass deposited (g); A is the piezoelectrically active area (area of electrode surface), = 0.126 cm^2; μ_q is shear modulus of quartz, = 2.947 x 10^{11} g/cm^2; and ρ_q is density of quartz, = 2.648 g/cm^3, or,

$$\Delta F = \frac{-2.3\times10^6 f_0^2 \Delta m}{A}$$

Where ΔF is the change in frequency (Hz), f_0 is the resonant frequency of the crystal (MHz), Δm is the mass deposited (g) and A is the area coated, = 0.126 cm^2. This equation is valid only for a rigid coating.

The amount of protein A on silver surface electrode can calculate follow by:

$$N_{\text{Albumin}} = \frac{\Delta m_{\text{Albumin}}}{MW_{\text{Albumin}}}$$

Where $N_{\text{Protein A}}$ is the amount of protein A on silver electrode surface, $\Delta m_{\text{Protein A}}$ is the mass of protein A deposited (g) and $MW_{\text{Protein A}}$ is the molecular weight of protein A, = 45 kDa.

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The amount of anti-human albumin on silver surface electrode can calculate follow by:

$$N_{Ab} = \frac{\Delta m_{Ab}}{MW_{Ab}}$$

Where N_{Ab} is the amount of anti-human albumin on silver electrode surface, Δm_{Ab} is the mass of anti-human albumin deposited (g) and MW_{Ab} is the molecular weight of anti-human albumin, = 146 kDa.

The amount of human albumin on silver surface electrode can calculate follow by:

$$N_{Albumin} = \frac{\Delta m_{Albumin}}{MW_{Albumin}}$$

Where $N_{Albumin}$ is the amount of anti-human albumin on silver electrode surface, $\Delta m_{Albumin}$ is the mass of anti-human albumin deposited (g) and $MW_{Albumin}$ is the molecular weight of anti-human albumin, = 66 kDa.

3.3.2 Quartz Crystal Surface Cleaning Comparison

The aim of quartz crystal surface preparation was to cleaned undesirable matter on the surface such as lipid, metal ion, or protein to get the fundamental frequency (F_0) with high accuracy. The comparison between two methods for cleaning silver surface of quartz crystal was performed. First method, the silver electrodes of crystal was cleaned with 30 % ethanol, and another one, the silver electrode was cleaned with PBS pH 7.4 and after cleaned with two methods each one was rinsed with distilled water. After leaved to dry about 2 hours in desiccators, the resonant frequency of the crystal was determined until a steady baseline was obtained and compared with control crystal that without cleaned. After that, all quartz crystals were applied protein A at 1.0 mg/ml, incubated overnight at 4 ^{o}C in moist chamber. After cleaned with PBS pH 7.4

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and distilled water, all quartz crystal leaved to dry about 2 hour in desiccators and determined the resonant frequency of quartz crystals.

3.3.3 Study of Oscillation Frequency Stability of Quartz Crystal with Silver Electrode

Four quartz crystals were assayed by protein A, anti-human albumin and human albumin followed by the method that mention above. All quartz crystal were leaved to dry in desiccators and took to measure the frequency change everyday. The record of frequency change and physical characteristic of quartz crystal were a key to evaluated the effect from oxidation of silver electrode surface of quartz crystal.

3.3.4 Study of Optimal Conditions for Microalbumin Detection

The optimal concentration and incubation time of protein A and anti-human albumin was studied to prepare an effective mass sensitive immunosensor for measurement of microalbumin as following:

3.3.4.1 Optimization of Protein A Coating Method

Protein A has been used as coating material for antibody immobilization. Protein A at different concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, 0.75, and 1.0 mg/ml were selected to study the optimal concentration. The method of coating protein A was shown in Fig.3.3. The concentration of protein A with maximum frequency change was chosen for optimal anti-human albumin immobilization.

The study of incubation time and temperature of protein A were performed. One mg/ml of protein A was applied on crystal surface and then incubated in moisture chamber at 4-8 ^{o}C and room temperature for different incubation time.

3.3.4.2 Optimization of Anti-Human Albumin Immobilization

The anti-human albumin at ranging from 0.005, 0.01, 0.02, 0.05, 0.10, 0.20, 0.50, and 1.00 mg/ml was selected to study the optimal concentration. The method of immobilized anti-human albumin was shown in Fig.3.5. The value of anti-

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human albumin concentration providing the best dose response curve for microalbumin detection was chosen for using as microalbumin immunosensor.

Thence, the study of incubation time of antibody was carrying out. Incubation time of antibody at 1 min, 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, and 4 h. was selected to study the optimal incubation time of anti-human albumin.

3.3.5 Study of Dose Response Curve of Microalbumin by Piezoelectric Immunosensor

The optimum concentration and incubation time of protein A and anti-human albumin were chosen for microalbumin immunosensor preparation. The human albumin at ranging from 0.0001, 0.00025, 0.0005, 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, and 0.1 mg/ml was selected to study the response curve of microalbumin detection. The method of study response curve of microalbumin detection was shown in Fig.3.6. The dose response curve of microalbumin detection was demonstrated by plotting the frequency change as a function of target concentration. The control group of this study was bare quartz crystal and quartz crystal that applied only protein A, applied protein A and anti-human albumin, and applied protein A, anti-human albumin and globulin protein (IgG) as negative control.

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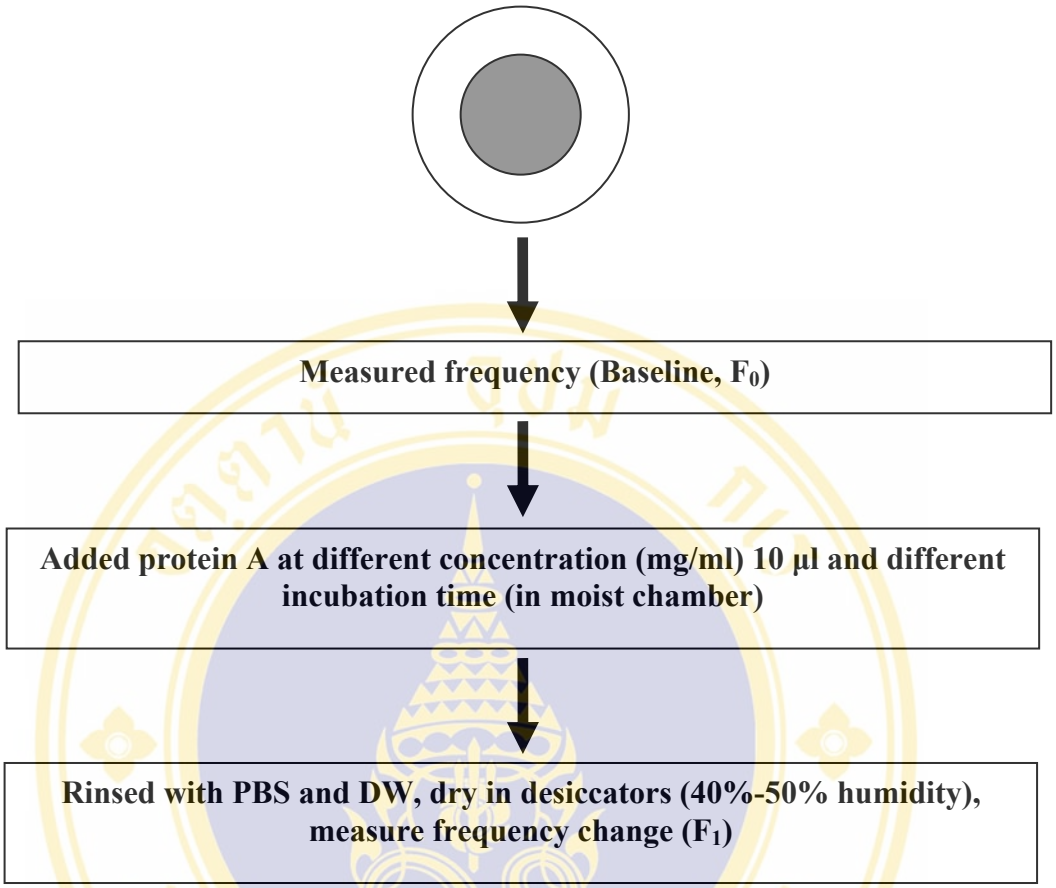

Figure 3.3 Schematic Diagram of Protein A Coating Optimization Study.

Crystals were measured baseline frequency of quartz crystal (F_0). Then, added protein A 10µl followed upon ranging from 0.025, 0.05, 0.1, 0.25, 0.5, 0.75, and 1.0 mg/ml, respectively (n=3), incubated overnight in moist chamber. Next, rinsed with PBS, distilled water and dried at desiccators (40%-50% of humidity). Finally, measured the frequency shift (Hz) (F_1-F_0). Then, the optimum concentration of protein A was chosen to study the optimum incubation time and temperature of protein A by incubated in ranging from 1 min, 5 min., 15 min., 30 min., 1 h., 2 h., 4 h., 6 h., 12 h., and overnight in refrigerator and 10 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 16 h at room temperature (n=3).

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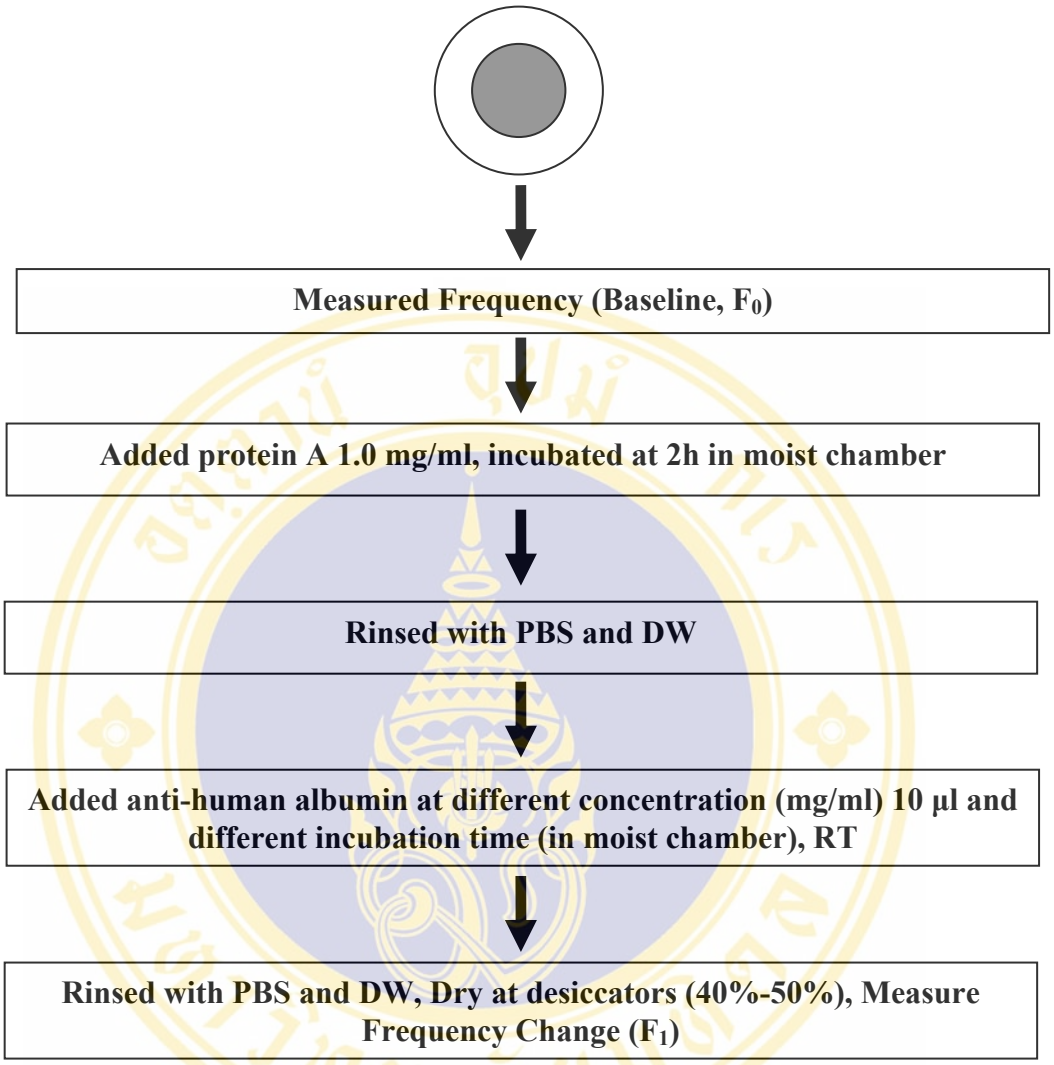

Figure 3.4 Schematic Diagram of Anti-Human Albumin Optimization Study

Crystals were measured fundamental frequency of quartz crystal (F_0). Then, added protein A 1.0 mg/ml, 10µl incubated 2 h in moist chamber, RT. Next, rinsed with PBS and distilled water. Then, added anti-human albumin at ranging from 0.005, 0.01, 0.02, 0.05, 0.10, 0.20, 0.50, and 1.00 mg/ml, respectively (n=3), 10 µl incubated 2 h in moist chamber, RT. After that, rinsed with PBS and distilled water, dried in desiccators (40%-50%). Finally, they measured the frequency shift (Hz) (F_1). Then, the optimum concentration of anti-human albumin was chosen to study the optimum incubation time of anti-human albumin by incubated in ranging from 1 min, 5 min., 15 min., 30 min., 1 h., 2 h., 3 h., and 4 h, respectively (n=3).

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3.3.6 Study of Binding Capability of Anti-Human Albumin on Quartz Crystal Electrode Surface After Storage

Fourteen groups of quartz crystal were assayed by protein A and anti-human albumin followed by the method that mention above. All quartz crystal were kept in cold room (4 ^{o}C – 6 ^{o}C) before use. Each group of quartz crystal were taken to applied human albumin 0.1 mg/ml in different storage time at ranging from 0, 2, 4, 6, 8, 22, 24, 26, 30, 37, 44, 51, 58, and 65 day. The measurement of frequency change was performed.

3.3.7 Comparative Study of Microalbumin Detection in Urine Sample between Mass Sensitive Immunosensor and Conventional System

The conventional method used to measure microalbumin level relies on an early morning urine collection. The microalbumin test is performed by the Hitachi 917 used for routine work in almost clinical laboratory. Albumin in urine sample agglutinated with latex particles that coated with anti-human albumin. The agglutination of the particles is proportional to the microalbumin concentration and can be measured by turbidimetry at 340 nm (primary) and 700 nm (secondary).

After the calibration curve was generated by plotting frequency change against various human albumin concentrations, the detection limit was evaluated. Then, the clinical samples were detected individually with mass sensitive immunosensor (QCM) and immunoturbidimetry (Hitachi 917). Results were analyzed with correlation curve, correlation analysis, and paired samples (carried out by SPSS, version 13).

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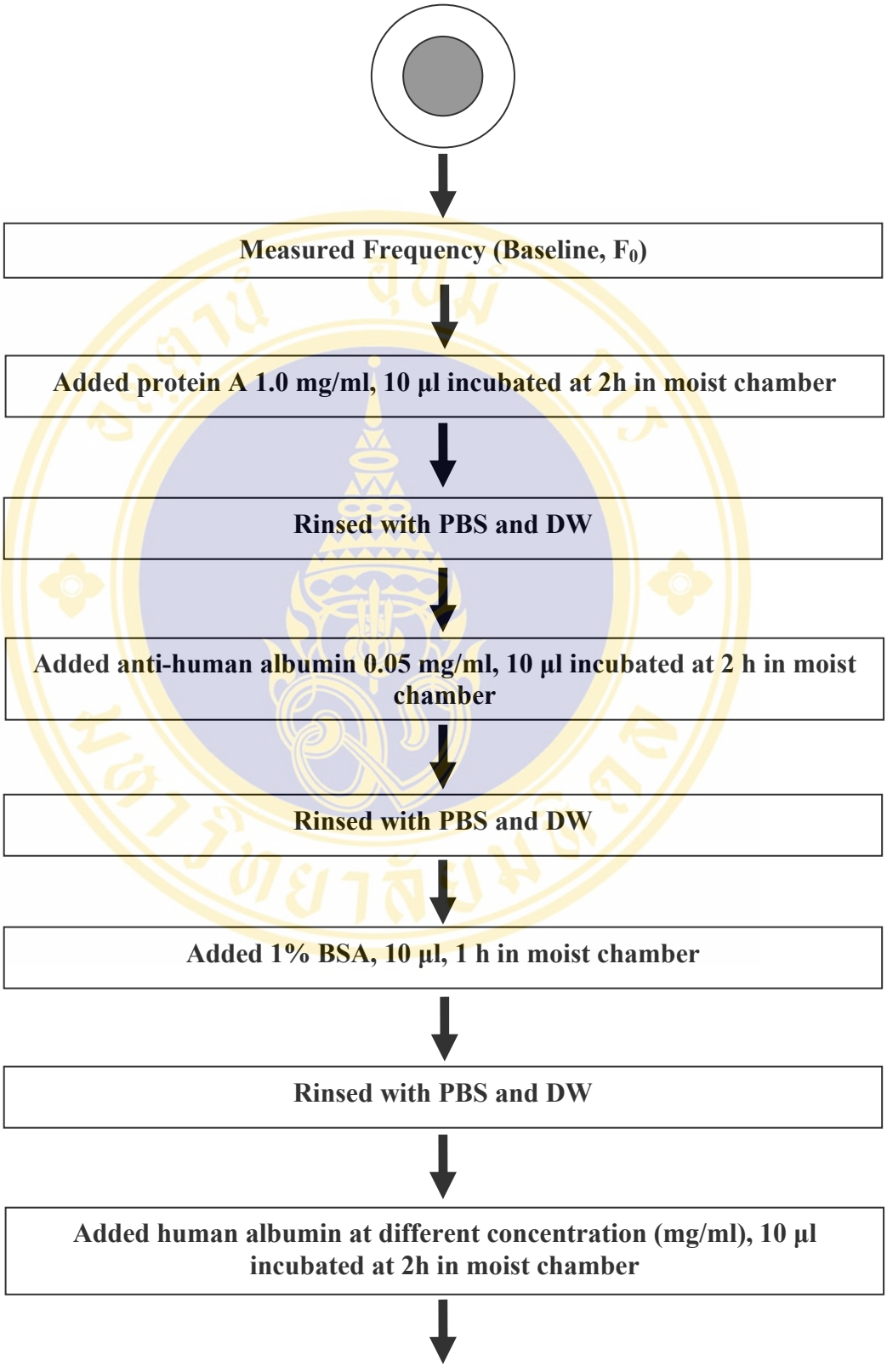

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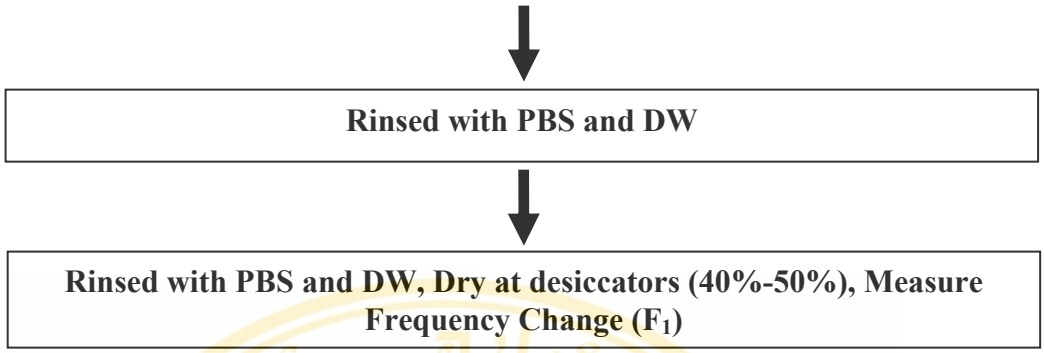

Figure 3.5 The Method of Dose Response Study for Microalbumin Determination

Crystals were measured fundamental frequency of quartz crystal (F_0). Then, added protein A 1.0 mg/ml, 10 µl incubated 2 h in moist chamber. Next, rinsed with PBS and distilled water. Then, added anti-human albumin 0.05 mg/ml, 10 µl incubated 2 h in moist chamber. After that, rinsed with PBS and distilled water. Thence, added 1% BSA 10 µl incubated 1 h in moist chamber, rinsed with PBS and distilled water, and added human albumin at ranging from 0.0001, 0.00025, 0.0005, 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, and 0.1 mg/ml, respectively (n=3), 10 µl incubated 2 h in moist chamber, and rinsed with PBS and distilled water, dried in desiccators (40%-50%). Thence, they measured the frequency shift (Hz) (F_1).

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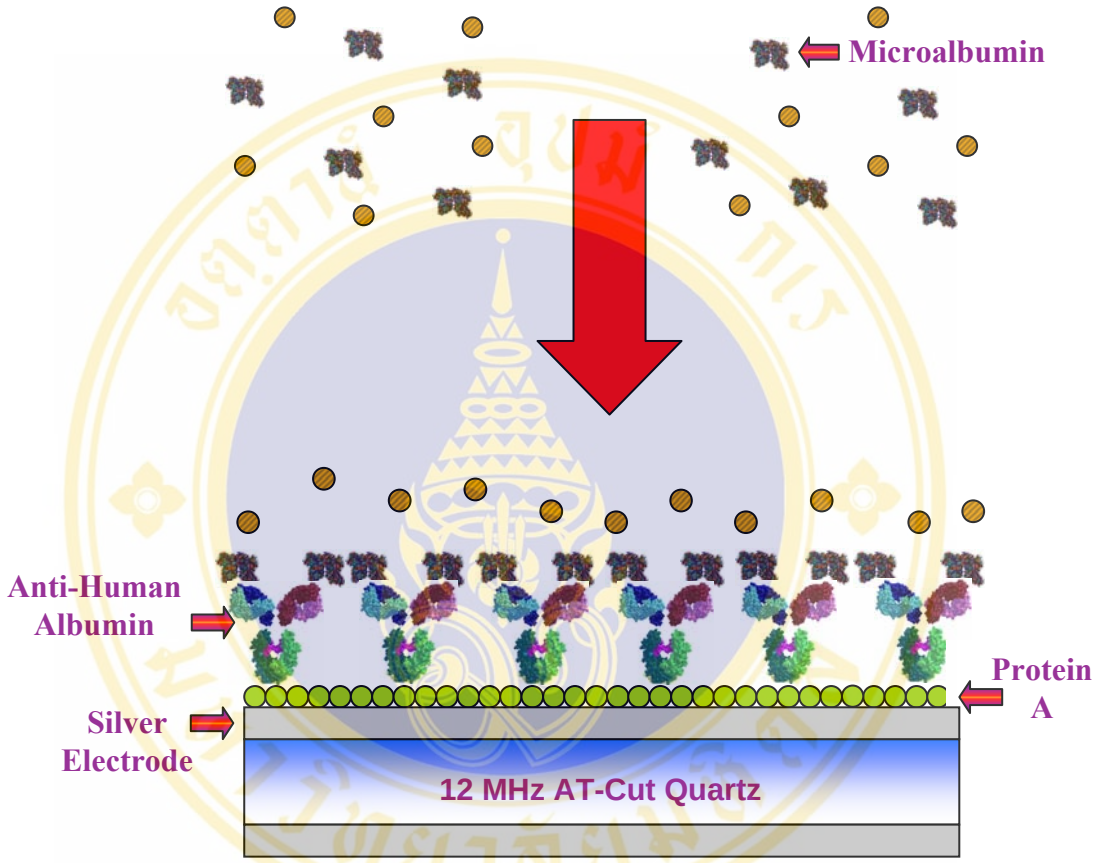

Figure 3.6 Schematic Diagram of Piezoelectric Immunosensor

Protein A was coated on the silver electrode quartz surface. Next, anti-human albumin was immobilized; F_c of anti-human albumin binds to protein A and F_{ab} adhere specifically to microalbumin.

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CHAPTER IV
RESULTS

4.1 Quartz Crystal Surface Preparation

The comparison between two methods for cleaning silver surface of quartz crystal was occurred. After cleaned with two methods, 30 % ethanol and PBS pH 7.4, the resonant frequency was determined and compared with control group that not cleaned. It was found that the frequency shift of three groups did not different. After that, all quartz crystals were determined the frequency shift after coated with protein A 1.0 mg/ml. The average frequency shift of quartz crystal was 191 ± 4.4 Hz after coating with protein A. The mass change that calculated from frequency shift of protein A at 1.0 mg/ml was 72.5 ng and has 9.7×10^{11} molecules of protein A on silver electrode surface. It was found that the frequency shift of three groups gave the same result ($P > 0.01$) (Fig. 4.1).

4.2 Oxidation Effect on Silver Electrode Surface of Quartz Crystal

Four quartz crystals were assayed by protein A, anti-human albumin and human albumin followed by the method that mention above. All quartz crystal were leaved to dry in desiccators and took to measure the frequency change everyday. The result of oxidation effect is shown in Figure 4.11. It was found that the frequency of all quartz crystals progressively decrease every day (mean = -3.175 Hz/day) and after 20 days the tendency of frequency to be stable. The change of color of the silver surface electrode was occurred in second week.

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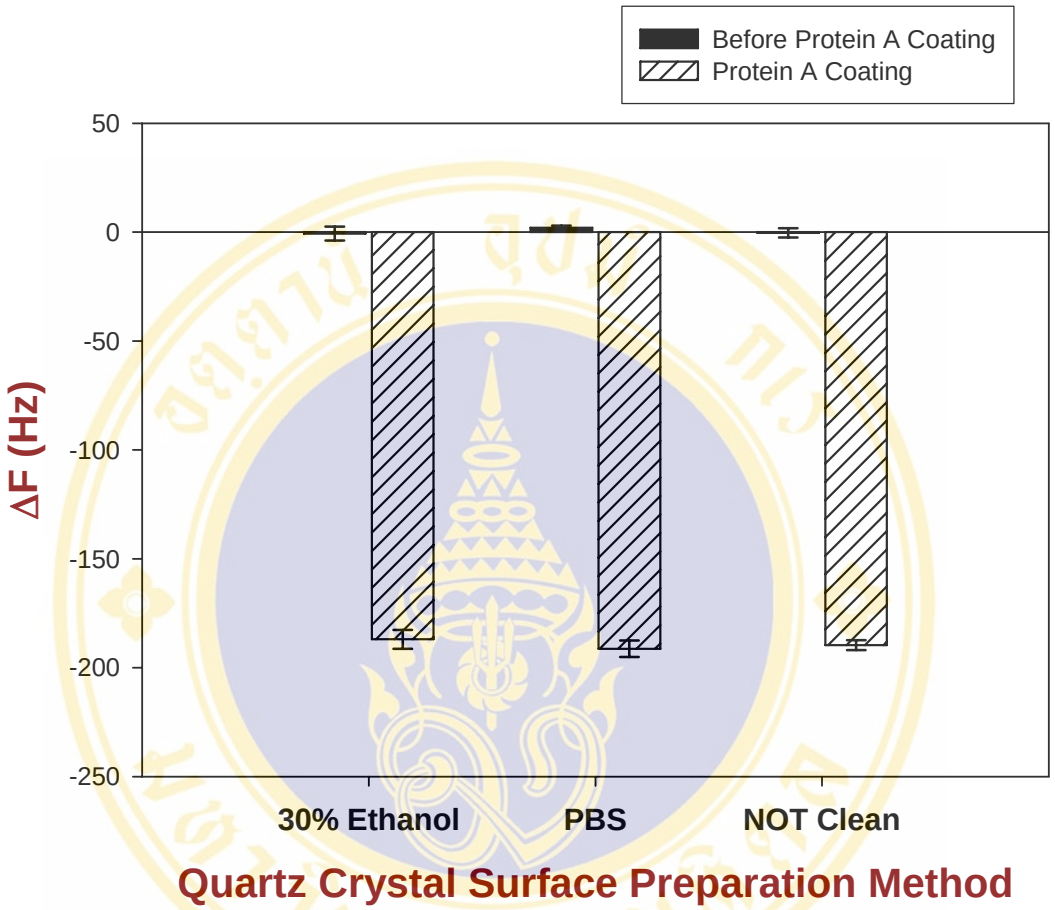

Figure 4.1 Optimization of Quartz Crystal Surface Preparation

Quartz crystals were cleaned with two methods for cleaning silver surface. After cleaned with two methods, 30 % ethanol and PBS pH 7.4, the resonant frequency was determined and compared with control group that not cleaned (n=3). It was found that the frequency shift of three groups did not different. After that, all quartz crystals were determined the frequency shift after coated with protein A 1.0 mg/ml, incubated 2 h in moist chamber at RT, washed with PBS pH 7.4 and distilled water, dried in desiccators (40%-50%). The frequency shift of three groups was not significant different ($P > 0.01$).

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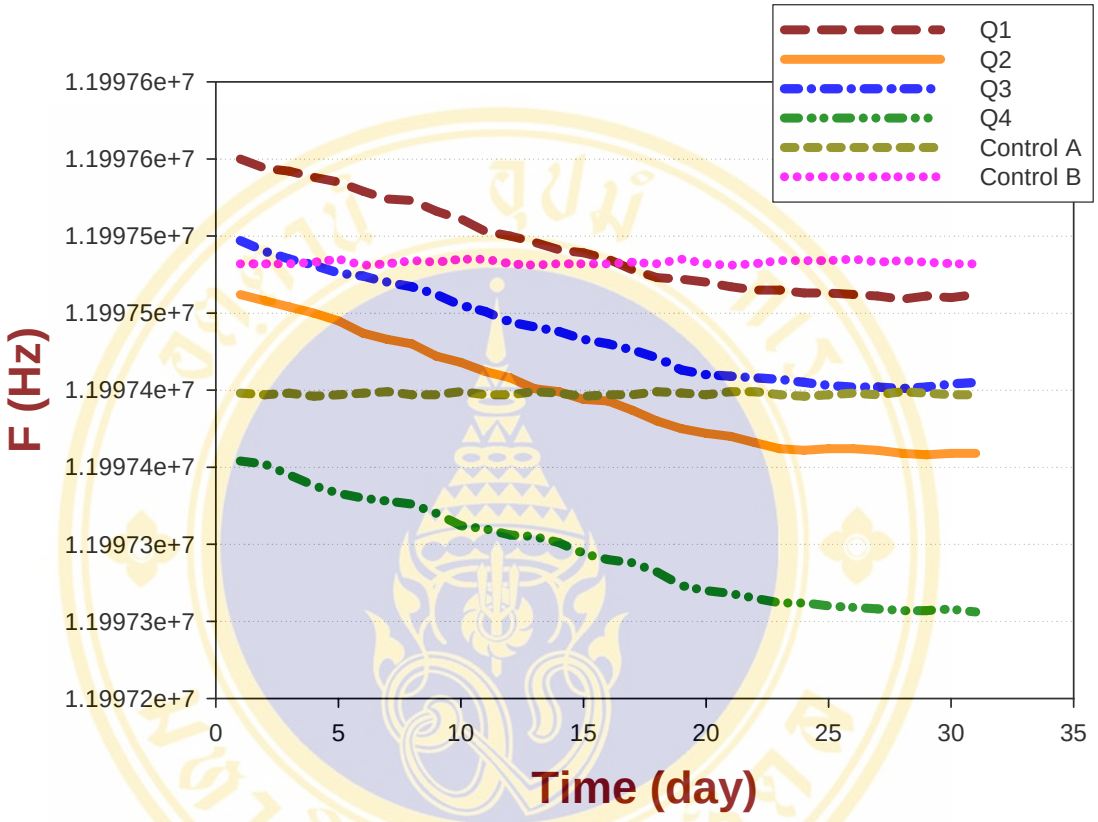

Figure 4.2 Response of Oxidation Effect on Silver Electrode Surface of Quartz Crystal

Four quartz crystals were assayed by protein A (1.0 mg/ml), anti-human albumin (0.05 mg/ml) and human albumin (1.0 mg/ml). All quartz crystal were leaved to dry in desiccators and took to measure the frequency change everyday. It was found that the frequency of all quartz crystals progressively decrease every day (mean = -3.175 Hz/day) and after 20 days the tendency of frequency to be stable.

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4.3 Study of Optimal Conditions for Microalbumin Detection

4.3.1 Optimization of Protein A for Anti-Human Albumin Immobilization

First, the optimum amount of protein A on the silver surface of quartz crystal was determined for anti-human albumin immobilization. The result of protein A immobilization at different concentration is shown in Figure 4.2. It was found that the frequency shift of protein A at different concentration ranging from 0.025, 0.05, 0.1, 0.25, 0.5, 0.75 and 1.0 mg/ml were -137, -153.67, -171, -185.33, -204.33, -205.33, and -206 Hz, respectively. The highest of signal was constant when 0.5 mg/ml. One mg/ml of protein A was selected and used throughout the study. The mass change that calculated from frequency shift of protein A 1.0 mg/ml was 78.2 ng and has 1.05×10^{12} molecules of protein A on silver electrode surface.

Thence, the study of incubation time of protein A was occurred. The result of optimum incubation time of protein A is shown in Figure 4.3 and 4.4. It was found that in the group of incubated in refrigerator the highest signal was constant after 6 hours and in the group of incubated at room temperature the highest signal was constant when 2 hours. Two hours of incubation time of protein A at room temperature was selected and used throughout the study.

4.3.2 Optimization of Anti-Human Albumin Immobilization

The optimum amount of key reaction partner, the anti-human albumin was also determined to obtain the microalbumin detection. The result of anti-human albumin concentration is shown in Figure 4.5. It was found that the frequency shift of anti-human albumin that ranging from 0.005, 0.01, 0.02, 0.05, 0.10, 0.20, 0.50, and 1.00 mg/ml were -203.33, -248.33, -294.67, -359.67, -428.33, -477.67, -507, -502.67 Hz, respectively. The highest of signal was constant when 0.5 mg/ml. The concentration of anti-human albumin 0.5 mg/ml was selected and used throughout the study. The mass change that calculated from frequency shift of anti-human albumin at 0.5 mg/ml was 192.4 ng and has 7.94×10^{11} molecules of anti-human albumin on silver electrode surface.

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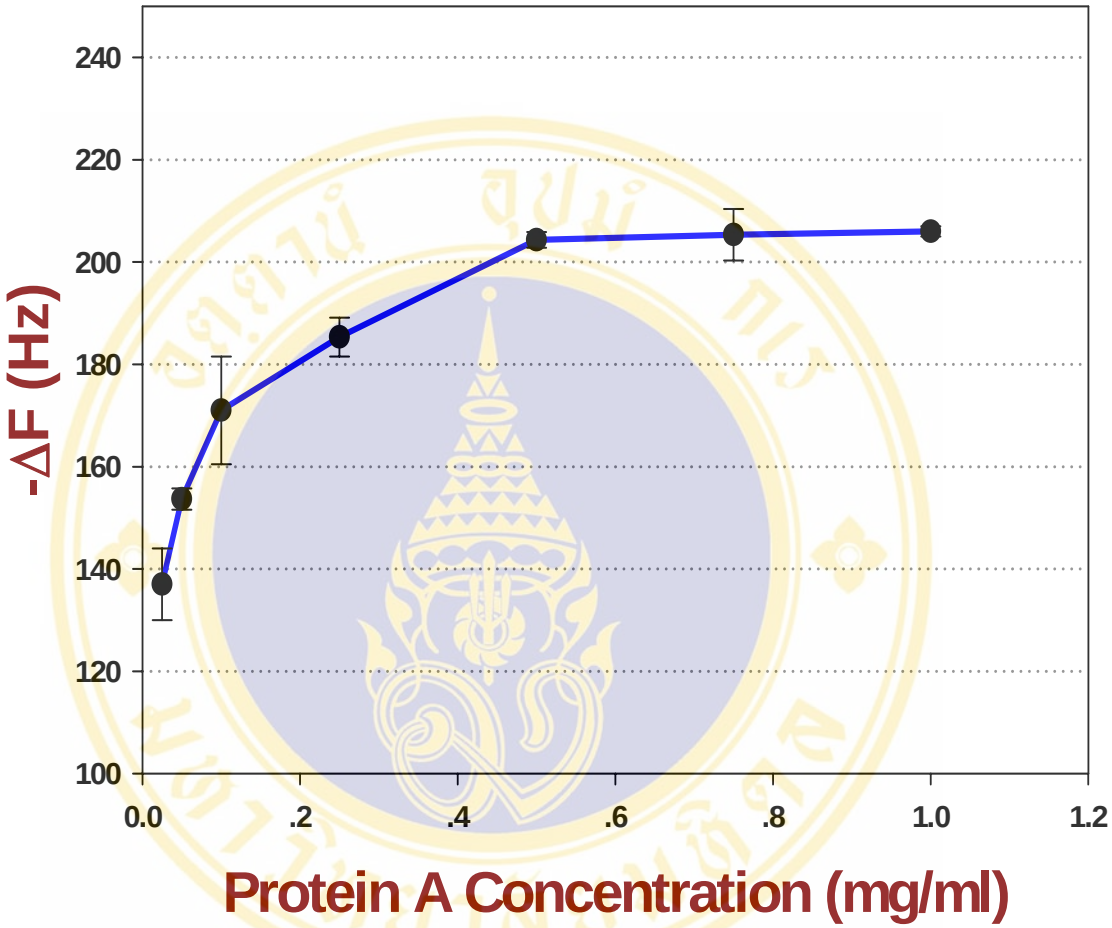

Figure 4.3 Optimization of Protein A Immobilization on Silver Electrode Surface of Quartz Crystal

Protein A at concentration of 0.025, 0.05, 0.1, 0.25, 0.5, 0.75 and 1.0 mg/ml were immobilized on the silver electrode surface of quartz crystal at 4 ^{o}C for overnight in moist chamber. The resonant frequency was measured after quartz washed and dried. Values are mean ± S.D. (n=3). The result shown that the highest signal was constant when 0.5 mg/ml. The frequency shift was -204.33 Hz (± 1.53).

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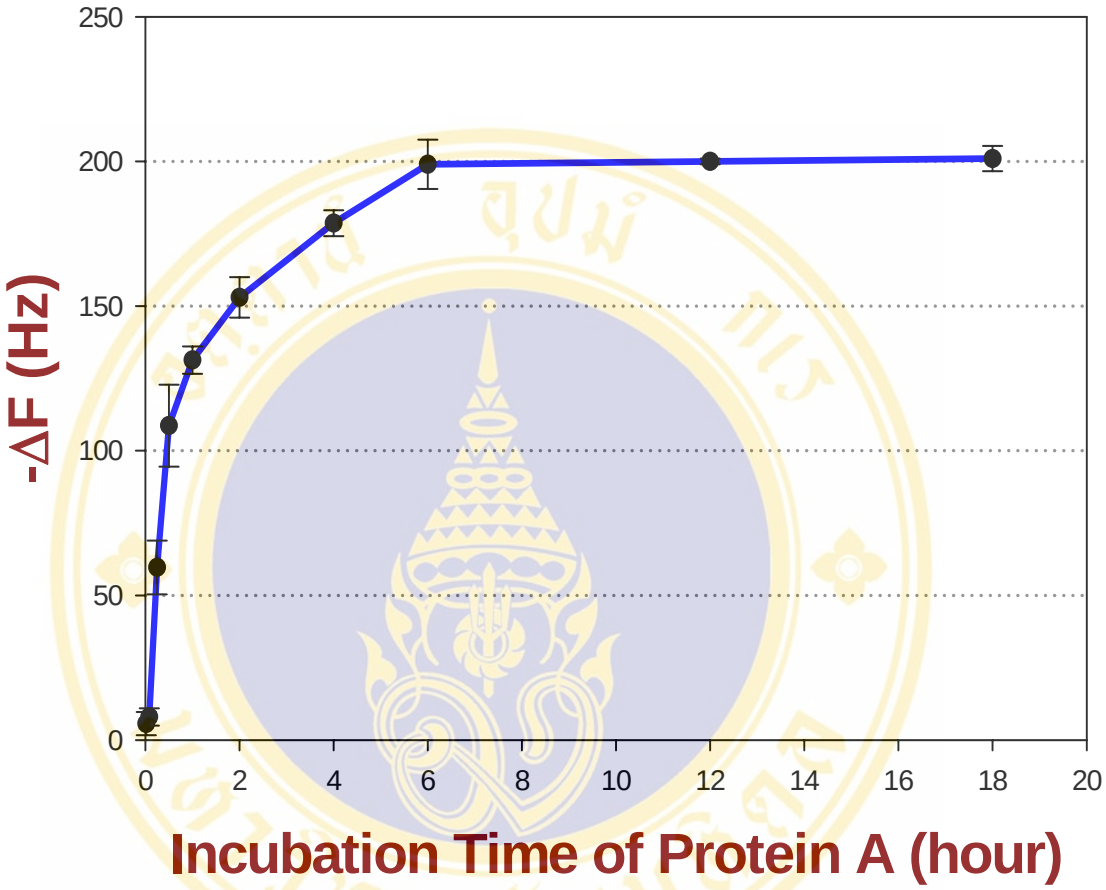

Figure 4.4 Optimization of Protein A Incubation Time at Refrigerator (4-8 ^{o}C)

Protein A 1.0 mg/ml was coating on the silver electrode surface of quartz crystal at different incubation time from 1 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 12 h, and overnight in moist chamber on refrigerator (4-8 ^{o}C). The resonant frequency was measured after quartz washed and dried. Values are mean ± S.D. (n=3). The result shown that the highest signal was constant after 6 hours. The frequency shift was -199 Hz (± 8.54).

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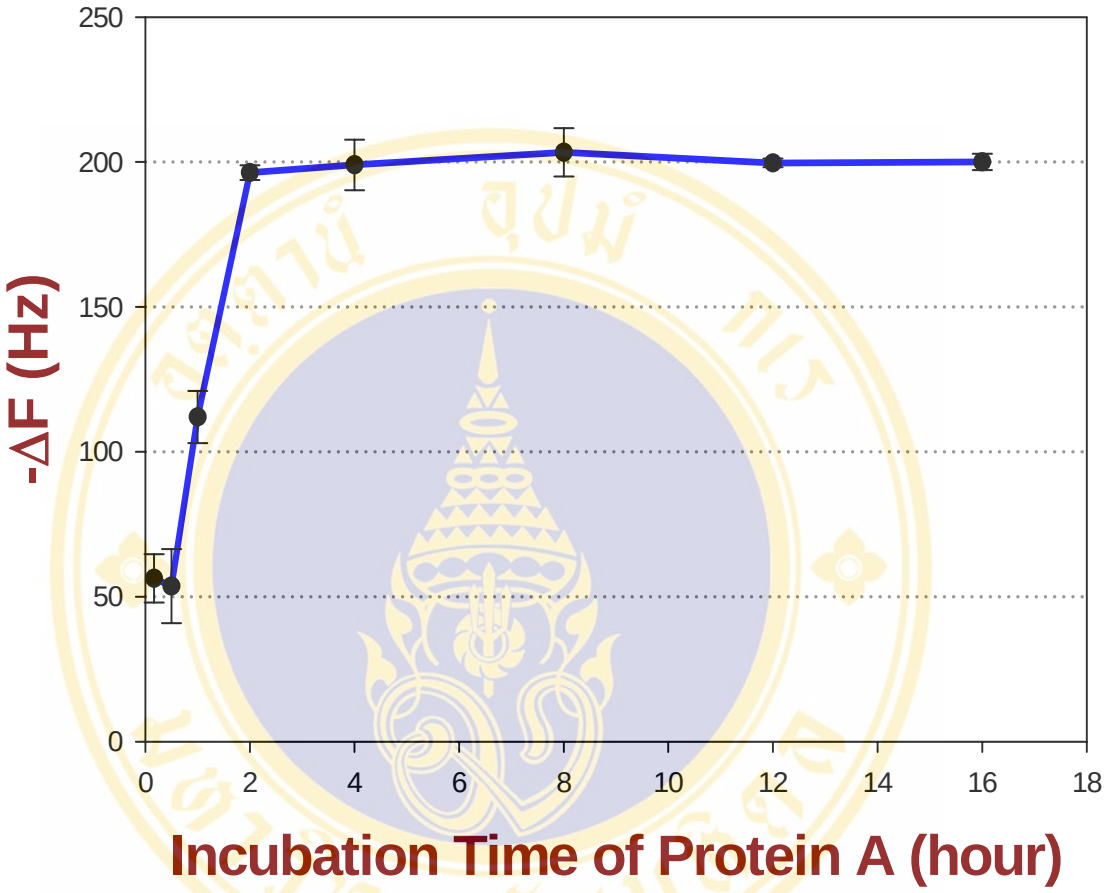

Figure 4.5 Optimization of Protein A Incubation Time at Room Temperature

Protein A 1.0 mg/ml was coating on the silver electrode surface of quartz crystal at different incubation time from 10 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 16 h in moist chamber at room temperature. The resonant frequency was measured after quartz washed and dried. Values are mean ± S.D. (n=3). The result shown that the highest signal was constant after 2 hours. The frequency shift was -196.33 Hz (± 2.52).

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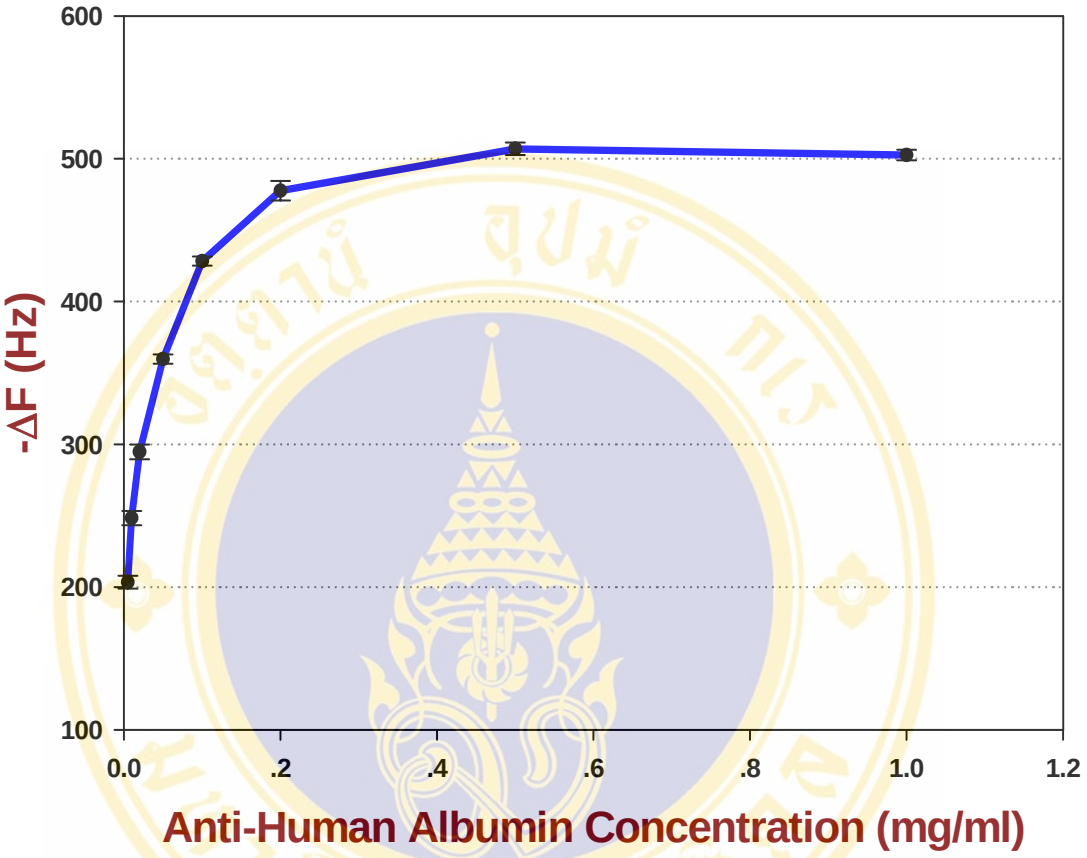

Figure 4.6 Optimization of Anti-Human Albumin Immobilization on Silver Electrode Surface of Quartz Crystal

Anti-human albumin at concentration of 0.005, 0.01, 0.02, 0.05, 0.10, 0.20, 0.50, and 1.00 mg/ml were immobilized on the silver electrode surface with protein A 1.0 mg/ml at RT for 2 h in moist chamber. The resonant frequency was measured after washed and dried. Values are mean ± S.D. (n=3) and subtract from the frequency shift of protein A. The highest signal was obtained when 0.5 mg/ml. The frequency shift was -507 Hz (± 4.36).

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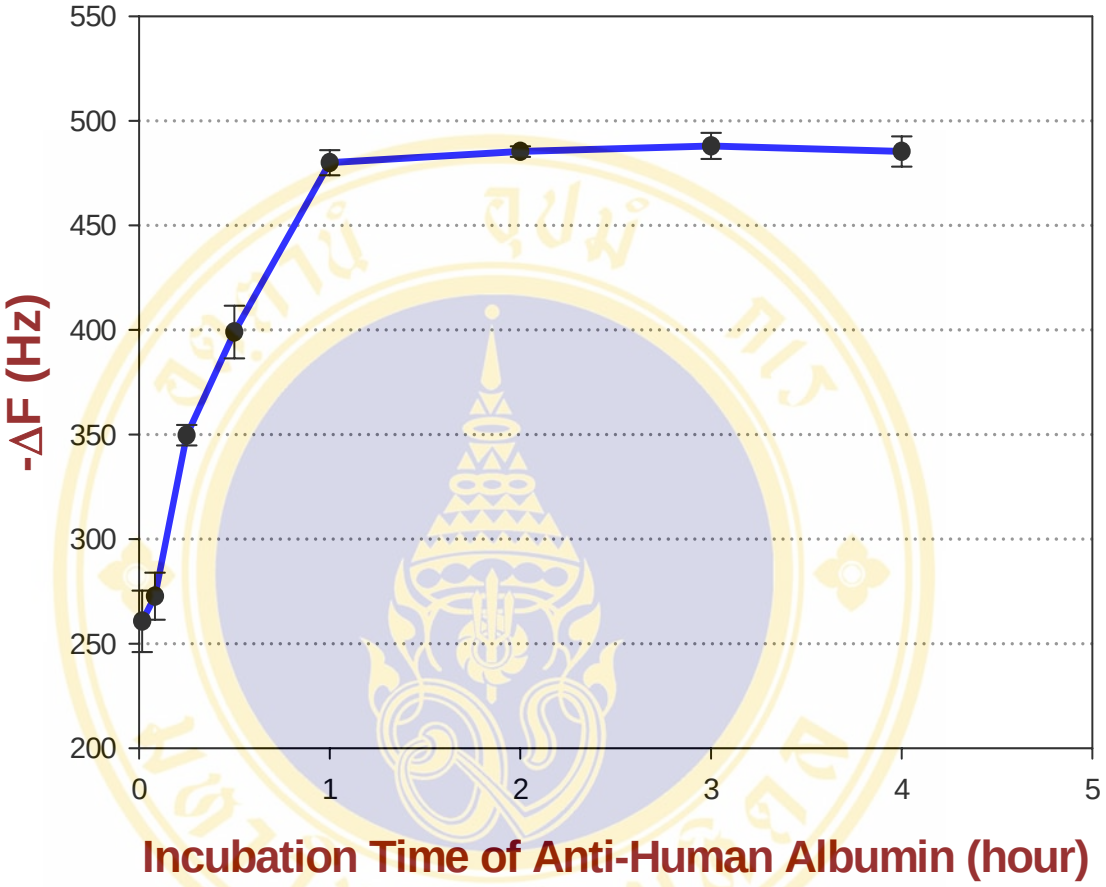

Figure 4.7 Optimization of Anti-Human Albumin Incubation Time at Room Temperature.

Anti-human albumin 0.5 mg/ml was coating on the silver electrode surface with protein A 1.0 mg/ml at different incubation time from 1 min, 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, and 4 h in moist chamber at room temperature. The resonant frequency was measured after washed and dried. Values are mean ± S.D. (n=3) and subtract from the frequency shift of protein A. The highest signal was constant when 1 hour. The frequency shift was -480 Hz (± 6).

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After that, the study of incubation time of anti-human albumin was occurred. The result of optimum incubation time of anti-human albumin is shown in Figure 4.6. It was found that the highest signal was constant when 1 hour. Two hours of incubation time of anti-human albumin at room temperature was selected and used throughout the study.

4.4 Dose Response Study of Microalbumin by Piezoelectric Immunosensor

One mg/ml of protein A was coated on the silver surface of electrode and immobilized by 0.5 mg/ml of anti-human albumin that it gave the maximum frequency shift in process of preparation microalbumin sensor. The main concept of this assay is based on the reaction between anti-human albumin and human albumin. The result of response curve is shown in Figure 4.7 and 4.8. It was found that the frequency shift of human albumin that ranging from 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1.0 mg/ml were -89.33, -126.33, -157, -175.33, -200.33, -250.33, -315.33, and -429.67 Hz, respectively. The linearity of curve shown the unwelcome result (R^2 = 0.904).

Ten µl of 0.05 mg/ml of anti-human albumin was immobilized on the silver surface after coated with 1.0 mg/ml of protein A to resolve the problem. The result of response curve is shown in Figure 4.9 and 4.10. It was found that the frequency shift of human albumin that ranging from 0.0001, 0.00025, 0.0005, 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, and 0.1 mg/ml were -87, -115.67, -137.33, -158, -187.33, -206, -230, -257.67, -279, and -300.67 Hz, respectively. The linearity of curve shown the pleasing result (R^2 = 0.999). This calibration curve was selected and used throughout the study. The detection limit that calculated from this curve was 9.5 ng/ml.

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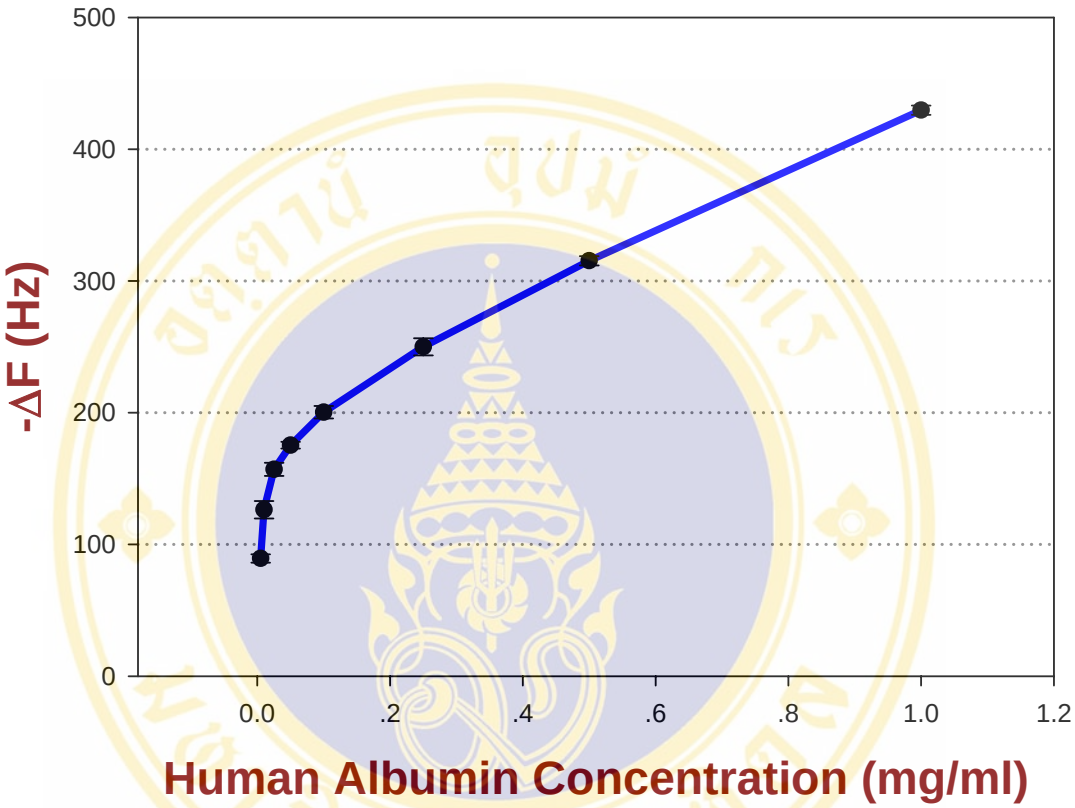

Figure 4.8 Dose Response Curve of Microalbumin Assay with Anti-Human Albumin on Quartz Crystal Surface ([Ab]=0.5)

Ten µl of human albumin that ranging from 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1.0 mg/ml were applied on the silver electrode surface with protein A (1.0 mg/ml) and anti-human albumin (0.5 mg/ml). The resonant frequency was measured after washed and dried. Values are mean ± S.D. (n=3) and subtract from the frequency shift of protein A and anti-human albumin.

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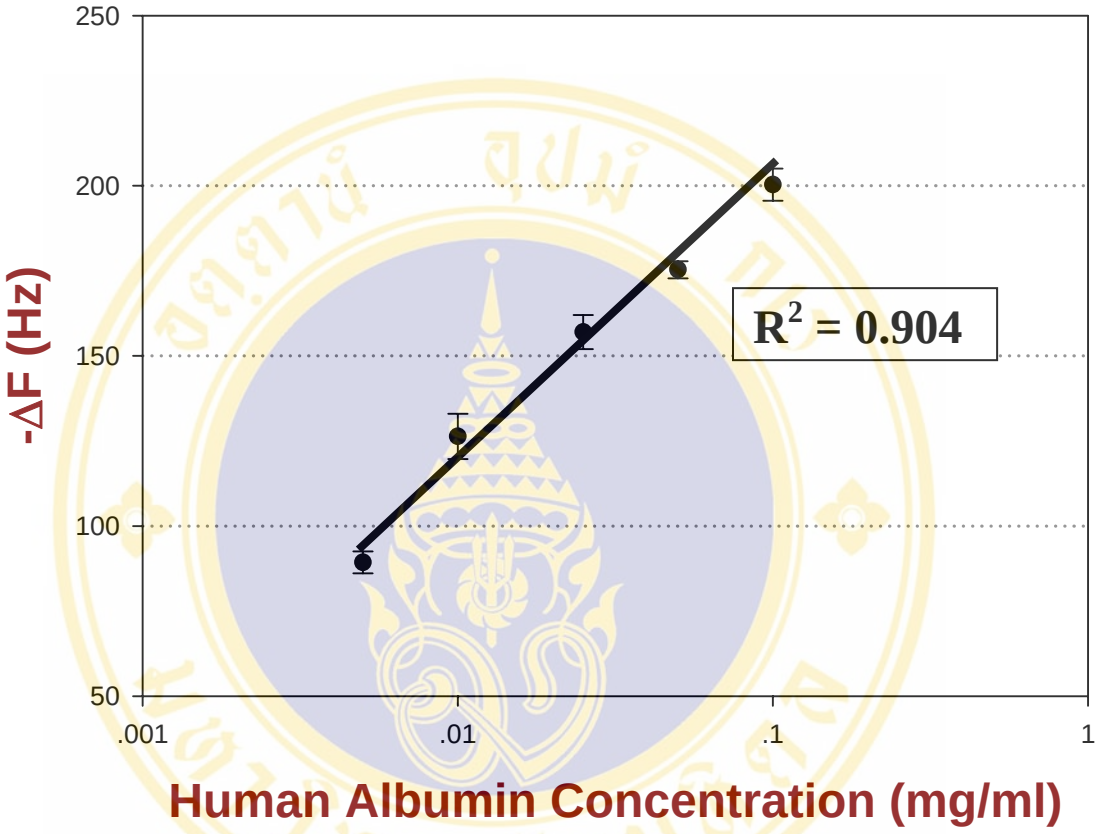

Figure 4.9 Calibration Curve of Microalbumin Assay with Anti-Human Albumin on Quartz Crystal Surface ([Ab]=0.5).

Ten µl of human albumin that ranging from 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1.0 mg/ml were applied on the silver electrode surface with protein A (1.0 mg/ml) and anti-human albumin (0.5 mg/ml). The resonant frequency was measured after washed and dried. Values are mean ± S.D. (n=3) and subtract from the frequency shift of protein A and anti-human albumin. The linearity of curve shown the unwelcome result (R^2 = 0.904).

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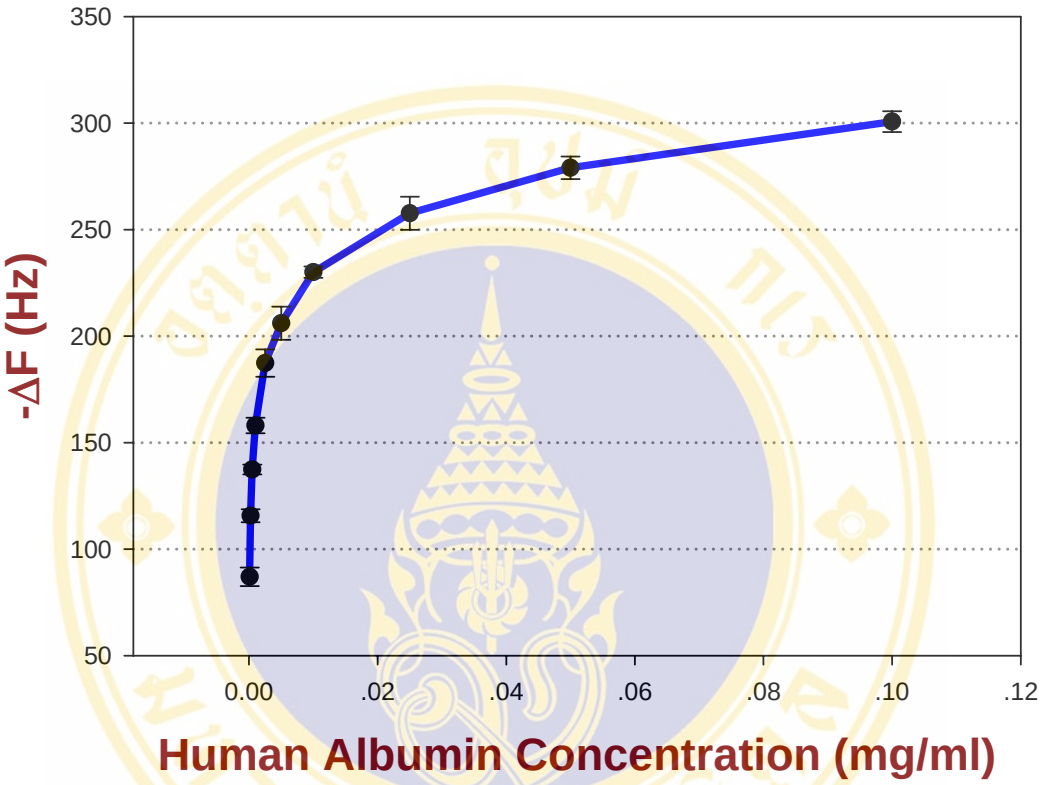

Figure 4.10 Dose Response Curve of Microalbumin Assay with Anti-Human Albumin on Quartz Crystal Surface ([Ab]=0.05)

Ten µl of human albumin that ranging from 0.0001, 0.00025, 0.0005, 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, and 0.1 mg/ml were applied on the silver electrode surface with protein A (1.0 mg/ml) and anti-human albumin (0.05 mg/ml). The resonant frequency was measured after washed and dried. Values are mean ± S.D. (n=3) and subtract from the frequency shift of protein A and anti-human albumin.

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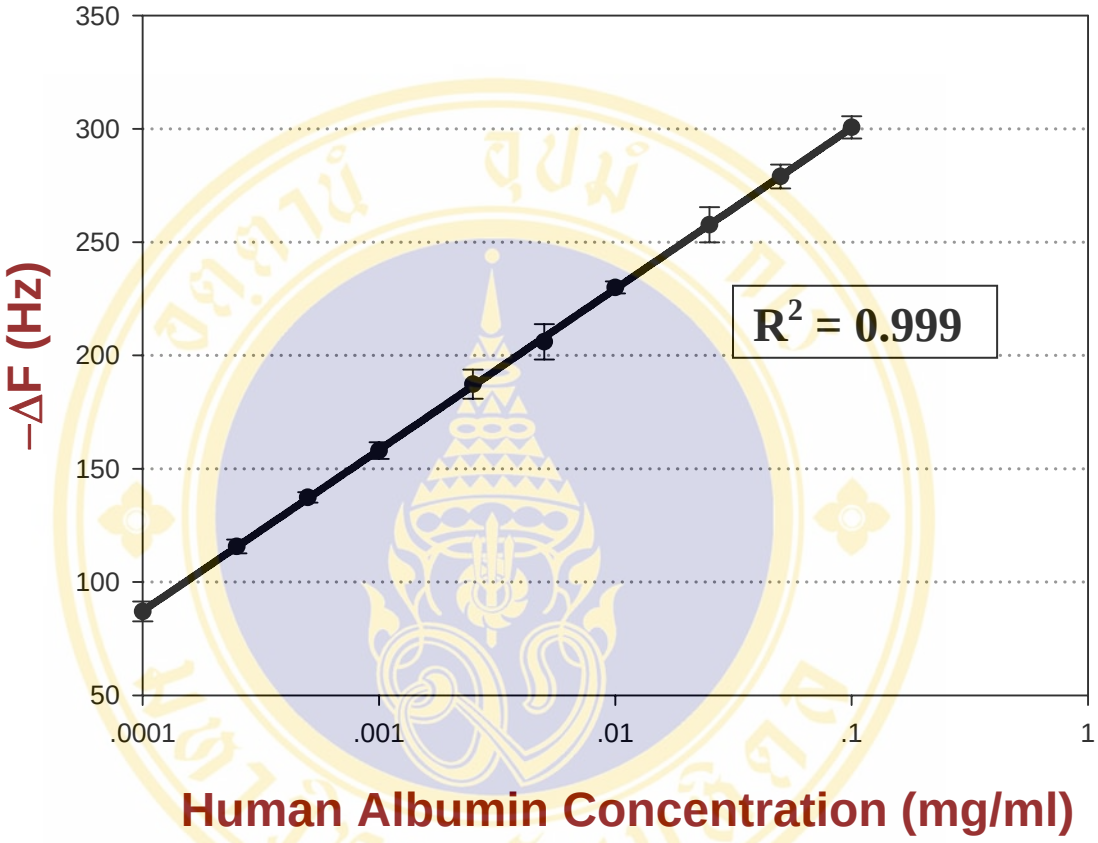

Figure 4.11 Calibration Curve of Microalbumin Assay with Anti-Human Albumin on Quartz Crystal Surface ([Ab]=0.05)

Ten µl of human albumin that ranging from 0.0001, 0.00025, 0.0005, 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, and 0.1 mg/ml were applied on the silver electrode surface with protein A (1.0 mg/ml) and anti-human albumin (0.05 mg/ml). The resonant frequency was measured after washed and dried. Values are mean ± S.D. (n=3) and subtract from the frequency shift of protein A and anti-human albumin. The linearity of curve shown the pleasing result (R^2 = 0.999).

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4.5 Study of Binding Capability of Anti-Human Albumin on Quartz Crystal Electrode Surface after Storage

Fourteen groups of quartz crystal were assayed by protein A and anti-human albumin. All quartz crystal were kept in cold room that control temperature in 4 ^{o}C – 6 ^{o}C before use. Each group of quartz crystal was taken to applied human albumin 0.1 mg/ml in different time of storage. The result of binding capacity of anti-human albumin on quartz crystal electrode surface after storage is shown in Figure 4.12. It was found that after storage 65 days the binding capability of antibody stilled work.

4.6 Comparative Study of Microalbumin Detection in Urine Sample between Mass Sensitive Immunosensor and Conventional System

After the calibration curve was generated by plotting frequency change against various human albumin concentrations, the detection limit was evaluated. Then, the clinical samples were detected individually with mass sensitive immunosensor (QCM) and immunoturbidimetry (Hitachi 917).

Sixty-four morning urine samples were collected from people who come for health check-ups at Center of Medical Laboratory Service and completely examined the protein value by strip test. Thirty-two urines with negative result of protein by strip test and 32 urines with positive result of protein by strip test were selected. The result of comparative study of microalbumin detection in urine sample between mass sensitive immunosensor and conventional system is shown in Figure 4.13. The correlation curve, correlation analysis and paired samples t-test analysis used to evaluate the correlation of two diagnostic methods for clinical sample detection. The correlation value was 0.999 and significantly correlates ($P < 0.01$). As well as, the result of paired samples t-test analysis between QCM and immunoturbidimetry was not significantly different ($P > 0.01$).

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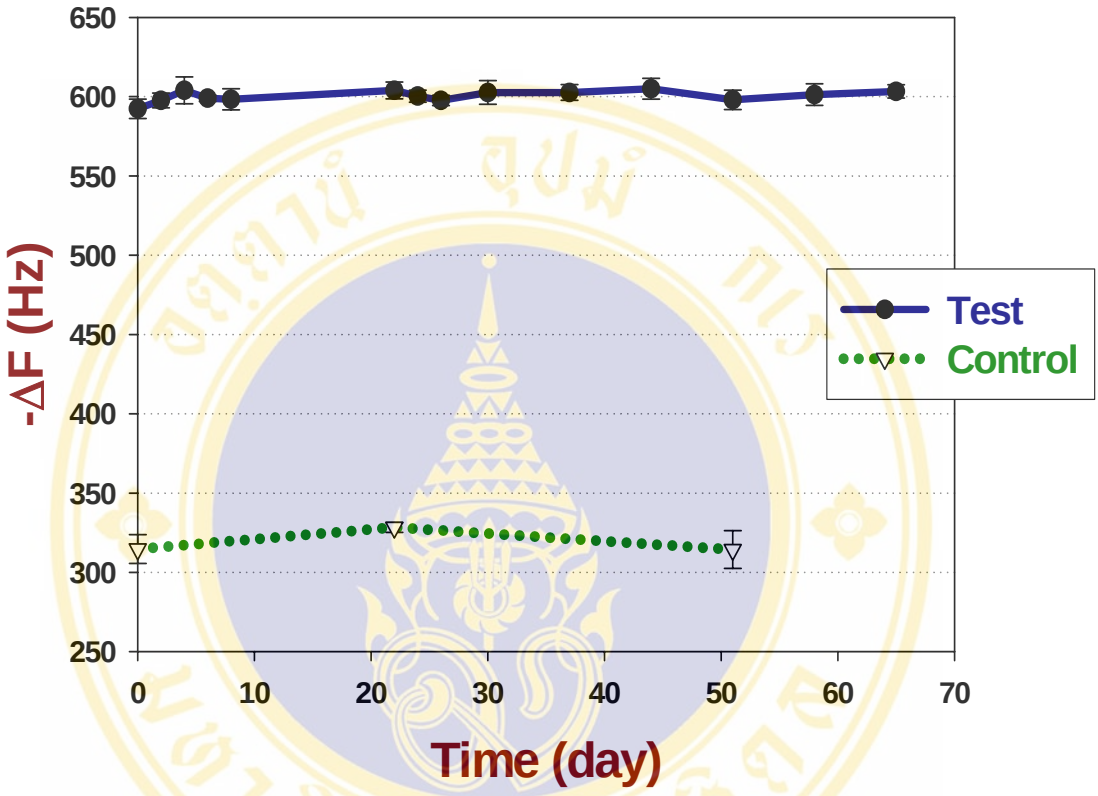

Figure 4.12 Binding Capability of Anti-Human Albumin on Quartz Crystal Electrode Surface After Storage

Fourteen groups of quartz crystal were assayed by protein A (1.0 mg/ml) and anti-human albumin (0.05 mg/ml). All quartz crystals were kept in cold room at 4 ^{o}C – 6 ^{o}C. Each group of quartz crystal were took to applied human albumin 0.1 mg/ml in different time of storage at ranging from 0, 2, 4, 6, 8, 22, 24, 26, 30, 37, 44, 51, 58, and 65 day. The control group of this study was quartz crystal that applied protein A and anti-human albumin without human albumin at different storage time from 0, 22, and 51 day. Values are mean ± S.D. (n=3) and subtract from the frequency shift of protein A. It was found that after storage 65 days the binding capability of antibody stilled work.

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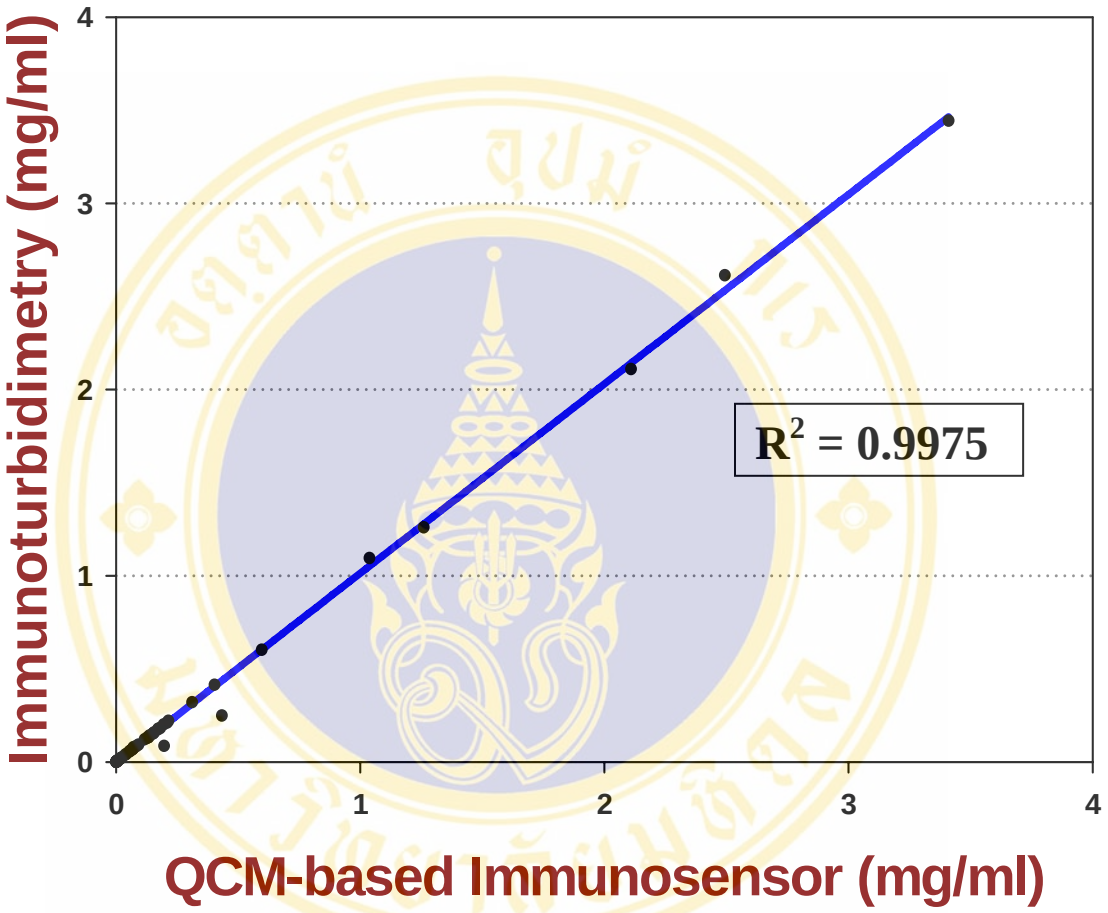

Figure 4.13 Correlation of Microalbumin Assay between QCM-based Immunosensor and Immunoturbidimetry

Sixty-four morning urine samples were collected from people who come for health check-ups. Thirty-two urines with negative result of protein by strip test and 32 urines with positive result of protein by strip test were selected. Thence, the clinical samples were detected individually with mass sensitive immunosensor (QCM) and immunoturbidimetry (Hitachi 917).

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The frequency change in each experiment was converted to mass change and amount of molecule on the silver electrode surface to evaluate reliability of the method. The example of calculated values in each experiment is shown in Table 4.1. The table shows frequency change, mass change, and amount of molecule of protein A at 1.0 mg/ml, anti-human albumin at 0.05 and 0.5 mg/ml, and human albumin at 0.1 mg/ml.

Table 4.1 Summary Calculated Value

Method		Protein A (1.0 mg/ml)	Anti-Human albumin		Human Albumin 0.1 mg/ml
			0.05 mg/ml	0.5 mg/ml	
Quartz Crystal Surface Cleaning Comparison	ΔF (Hz)	191	-	-	-
	Δm (ng)	72.5	-	-	-
	N (molecule)	9.7×10^{11}	-	-	-
Optimization of Protein A Coating Method	ΔF (Hz)	206	-	-	-
	Δm (ng)	78.2	-	-	-
	N (molecule)	10.5×10^{11}	-	-	-
Optimization of Anti-human albumin Immobilization	ΔF (Hz)	202	359	507	-
	Δm (ng)	76.7	136.2	192.4	-
	N (molecule)	10.3×10^{11}	5.6×10^{11}	7.9×10^{11}	-
Study of Dose Response Curve of Microalbumin by Piezoelectric Immunosensor	ΔF (Hz)	189	355	498	300
	Δm (ng)	71.7	134.7	189	114
	N (molecule)	9.6×10^{11}	5.6×10^{11}	7.8×10^{11}	10.3×10^{11}

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Study of Binding Capability of Anti-human Albumin on Quartz Crystal Electrode Surface After Storage	ΔF (Hz)	198	349	-	293
	Δm (ng)	75.2	132.4	-	111
	N (molecule)	10.1×10^{11}	5.5×10^{11}	-	10.1×10^{11}
Comparative Study of Microalbumin Detection in Urine Sample between Mass Sensitive Immunosensor and Conventional System	ΔF (Hz)	199	351	-	287
	Δm (ng)	75.5	133.2	-	109
	N (molecule)	10.1×10^{11}	5.5×10^{11}	-	9.9×10^{11}

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CHAPTER V
DISCUSSION

Kidney disease is classified as any disease or disorder that affects the function of the kidneys. If kidney lost ability to function healthy, waste product can build up to high levels in blood system. Then, kidney disease increases the risk of heart and blood vessel disease. Also, kidney disease may develop complication like high blood pressure, weak bones, anemia, nerve damage, and poor nutritional health. Chronic kidney disease is a major condition of kidney disease because the problem happens slowly over a long period of time but it is permanent damage. Early detection and treatment can keep or delay chronic kidney disease from change for the worse (1, 77-79).

Proteins can leak into urine when kidneys are damaged. The urinary proteins are more effective in sensitivity than blood urea nitrogen, blood creatinine, and uric acid for diagnosing introductory renal damage (6, 7, 80). The significance arise of microalbumin in urine is the predictor of clinical proteinuria and chronic kidney disease (29, 81-87). The prevalence of patients, who have protein negative result from urine dipstick test, have high level of microalbumin in urine about 6-40% depend on the cutoff value (88). Radio immunoassay, florescence based immunoassay, immunonephelometry, and immunoturbidimetry are the reference method and conventional methods to detect the urinary proteins but they have a many disadvantages and limitations (14-17, 19-21).

Immunosensor assay is as defined as analytical a device that has been developed for detection of antigen-antibody interaction on the surface of a device known as a transducer. Depending on the transducer technology manipulated, immunosensors can be divided into three principal classes: optical, electrochemical and piezoelectric (60-65, 70). The piezoelectric immunosensors, quartz crystal microbalance (QCM), is based on the measurement the changes of frequency response resulting from reaction of antigen-antibody onto a modified quartz crystal surface.

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The relationship between frequency shift and mass change for the QCM that measured in the air is given by the Sauerbrey equation (66).

The former research is shown the applications of piezoelectric immunosensors that used to determine the concentration of T-lymphocytes (89), erythrocytes (90), E. coli (91), *Staphylococcus aureus* (92), methamphetamine in human urine (93), human herpes (94), hepatitis (95), HIV (96), and IgM and C-reactive protein (97). Therefore, piezoelectric immunosensors are inexpensive, easy to use, may allow for wide screening, and can develop for a broad range of applications.

The piezoelectric quartz crystal with silver electrode and has a basic resonant frequency of 12 MHz are used through in this experiment. Firstly, the optimum preparation method of quartz crystal surface has been done. From previous study shown many methods to clean the silver surface of quartz crystal (98-100). For example, Mao Y., 2002, quartz crystal was immersed in NaOH solution and then rinsed with water and immersed in HCl solution follows by water. In this study, the resonant frequency shift was determined and compared between three groups of quartz crystal that is cleaned with 30% ethanol, PBS buffer pH 7.4, and original quartz crystal. The result was shown in Figure 4.1. The frequency shift of quartz crystal before and after protein A immobilized has no significant difference ($P > 0.01$). From these data, quartz crystal surface from the factory is not necessary to do any cleaning process before using as immunosensor.

The mostly research in a field of piezoelectric immunosensor used the gold to be the electrode surface. However, some previous study used the silver to be the electrode surface and used the polymer shielded to protect the effect from silver oxidation (105). In this study used the silver to be the electrode surface of quartz crystal because of the price is 10 times cheaper than the gold surface electrode. However, the oxidation of silver must be careful. Then, the study of oxidation effect on silver electrode surface of quartz crystals was operated in the next. Quartz crystals were assayed by protein A, anti-human albumin and human albumin followed by the method above mentioned and leaved to dry in desiccator. All of quartz crystals took to measure the frequency change everyday within 1 month. The result was shown in Figure 4.11. It was found that the frequency change of all quartz crystals progressively decrease every day (mean = -3.175 Hz/day) and after 20 days the tendency of

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frequency to be stable. The change of color of the silver surface electrode was occurred in second week. From these results, the effect from silver oxidation was only 0.6% of the experiment values and the experiment can be finished within a day. Therefore, the protection of silver oxidation was not necessary for this study.

The anti-human albumin was immobilized to silver surface electrode via protein A. Protein A, derived from the cell wall of *Staphylococcus aureus*, binds specific to the *Fc* region of various immunoglobulines. Protein A is a 42 kDa polypeptide that binds the residues in the CH2 and CH3 domains (C-terminal end) of immunoglobulin heavy chains. Protein A as chemical compound is a directed immobilization method due to its natural affinity towards the F_c region of immunoglobulines molecules. Thus, F_{ab} region of antibody can exposed itself to an environment which is suitable for interact with specific antigen (Fig. 5.1) (101, 102). The measurement of frequency shift of quartz crystals after coated with various concentration of protein A ranging from 0.025-1.0 mg/ml has been done. The result has shown the optimum concentration of protein A to assist the immobilization of anti-human albumin as in Figure 4.2. The highest frequency shift occurred when the concentration of protein A was 0.5 mg/ml and then the value was inclining to be constant. The concentration of protein A at 1.0 mg/ml was chosen and used throughout this study because of the certainty that protein A cover all of the area of silver electrode.

This result has shown similar with previous study that used protein A at concentration of 1.0 mg/ml (103, 104). The frequency shift at 1.0 mg/ml of protein A was -206 Hz (±1) and can be converted to mass change by Sauerbrey equation. The mass change that calculated from frequency shift of protein A 1.0 mg/ml was 78.2 ng or 1.05×10^{12} molecules of protein A on silver electrode surface. Afterward, the optimum incubation time of protein A was studied. The results were shown in Figure 4.3 and 4.4. The results were compared when the quartz crystals coated with protein A 1.0 mg/ml and incubated with different duration at different condition. First condition, quartz crystals were incubated with different duration ranging from 1 min–16 hours in refrigerator (4-8^{o}C) after coated with protein A 1.0 mg/ml. Second condition, quartz crystals were incubated with different duration ranging from 10 min–16 hours at room temperature (23-26^{o}C) after coated with protein A 1.0 mg/ml. The results showed that

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frequency shift of quartz crystals of first condition maximized when incubated 6 hours but for second condition was maximized when incubated only 2 hours. The higher temperature condition was not used in this study because of a previous study shown that a low temperature long-time association worked better for the non-covalent interaction between metal and protein A. It is likely that this association provided less energetic and more stable conditions for the molecules to interact, whereas at 37°C, the additional energy led to atomic vibrations and less stable interactions (101). However, the overmuch drop away of temperature have long period of incubation time to stable. Therefore, the incubation time of protein A at 2 hours, room temperature, was chosen as the optimum incubation time in this study.

The amount of anti-human albumin was also optimized. The optimum amount of anti-human albumin played a role in the effective of microalbumin detection. In this study, the amount of protein A was used at 1.0 mg/ml for absorption on the silver surface electrode before anti-human albumin immobilization.

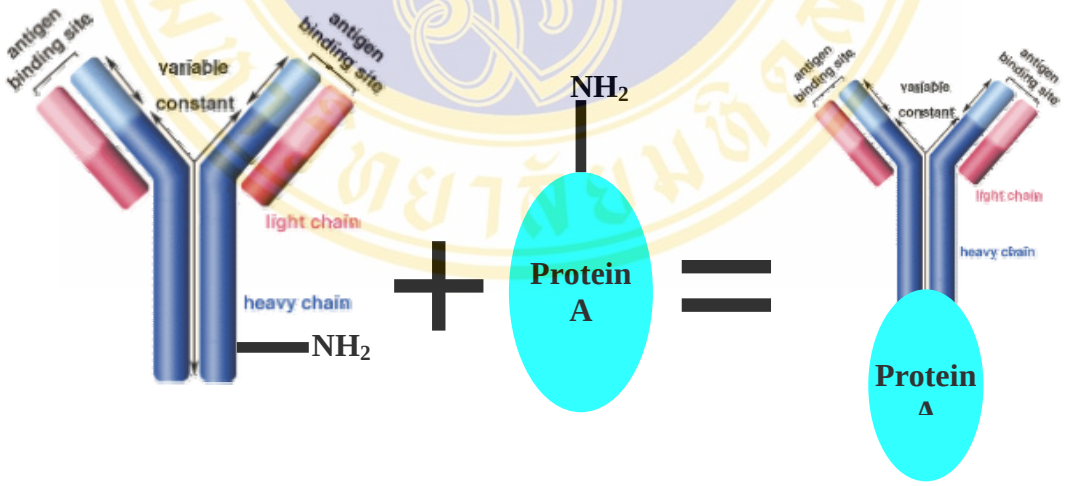

Figure 5.1 Binding of IgG and Protein A

Protein A, derived from the cell wall of *Staphylococcus aureus*, binds the Fc region of various immunoglobulines. Protein A is a 42 kDa polypeptide that binds the residues in the CH2 and CH3 domains of immunoglobulin heavy chains.

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Table 5.1 Determine Relative Avidity of Protein A for Polyclonal Antibodies of a Given Species (75, 76)

Species	Protein A, binding affinity
Human	++++
Mouse	++
Rat	+/-
Hamster	+
Guinea Pig	++++
Rabbit	++++
Horse	++
Cow	++
Pig	+++
Sheep	+/-
Goat	-
Chicken	-

The result was shown in Figure 4.5. The frequency shift in Figure 4.5 was shown only change of antibody. It was found that the frequency shift had proportionally related to the amount of anti-human albumin ranging from 0.005-1.0 mg/ml and reached the apex at 0.5 mg/ml and stable in higher concentration. It can be hypothesized that 0.5 mg/ml of anti-human albumin is an appropriate proportion for immobilization with 1.0 mg/ml of protein A. The frequency shift value at 0.5 mg/ml of anti-human albumin was -507 Hz (±4.36) and can be converted to mass change by Sauerbrey equation. The mass change that calculated from frequency shift of anti-human albumin at 0.5 mg/ml was 192.4 ng or 7.94×10^{11} molecules of anti-human albumin on silver electrode surface. The crude calculation of amount of anti-human albumin that can be fully immobilized on silver electrode surface (r = 2 mm) was 2×10^{12} molecules. Then, the amount of anti-human albumin at 0.5 mg/ml was not fully cover on the surface. The blocking reagent, bovine serum albumin (BSA), used to

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caulk between anti-human albumin molecules. However, some case of kidney disease such as multiple myeloma has fragment of immunoglobulin, especially IgG, into urine that can interact with free protein A. The commercial Fc fragment of immunoglobulin can used to be blocking material to resolve this problem. Afterward, the optimum incubation time of anti-human albumin was studied. The results were shown in Figure 4.6. Quartz crystals were incubated with different duration ranging from 1 min–4 hours at room temperature (23-26^{o}C) after coated with protein A 1.0 mg/ml and anti-human albumin 0.5 mg/ml. It's showed that frequency shift of quartz crystals of first condition maximized when incubated 2 hours. Therefore, the incubation time of anti-human albumin at 2 hours, room temperature, was chosen as the optimum incubation time in this study.

The study of dose response of microalbumin by piezoelectric immunosensor was continually operated. This study divided in to two sub-experiments. Firstly, the commercial human albumin, dissolved in PBS buffer pH 7.4, in different concentration ranging from 0.005-1.0 mg/ml was used as the target and applied on the surface of quartz crystals that coated with protein A 1.0 mg/ml and anti-human albumin 0.5 mg/ml before. The result was shown in Figure 4.7 and 4.8. It was found that the frequency shift had proportionally related to the amount of human albumin target and had a tendency to continually increase. The log-scale calibration curve shown the unwelcome result (R^2 = 0.904). It can be hypothesized that the overmuch of amount of anti-human albumin gave the steric effect and bothered the combination between antibody and antigen. To prove that assumption, the secondary sub-experiment was performed. The decreasing of concentration of anti-human albumin was hypothesized to resolve the problem and the focusing of amount of target that change to lesser value because of the condition of microalbuminuria was a little amount of protein leak in to urine. The commercial human albumin, dissolved in PBS buffer pH 7.4, in different concentration ranging from 0.0001-0.1 mg/ml was used as the target and applied on the surface of quartz crystals that coated with protein A 1.0 mg/ml and anti-human albumin 0.05 mg/ml before. The result was shown in Figure 4.9 and 4.10. It was found that the frequency shift had proportionally related to the amount of human albumin target and had a tendency to stable in higher concentration. The log-scale calibration curve shown the pleasing result (R^2 = 0.999). From these

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result, can demonstrated that the decreasing of concentration of anti-human albumin can be resolved the problem from steric effect of antibody because had more distance between molecules of antibody and can be increasing the amount of target to interacted with antibody. For example, in the point at 0.1 mg/ml of target, the frequency shift of firstly sub-experiment ([Ab] = 0.5 mg/ml) was -200.33 Hz (±4.7) and increased to -300.67 Hz (±4.9) for secondary sub-experiment ([Ab] = 0.05 mg/ml). The frequency shift value at 0.05 mg/ml of anti-human albumin was -359.67 Hz (±3.2) and can be converted to mass change by Sauerbrey equation. The mass change that calculated from frequency shift of anti-human albumin at 0.05 mg/ml was 136.5 ng or 5.63×10^{11} molecules of anti-human albumin on silver electrode surface. If the anti-human albumin has completely combined with target, the expected amount of human albumin was 1.12×10^{12} molecules but, from secondary sub-experiment at 0.1 mg/ml of human albumin, the frequency shift was -300.67 Hz (±4.9) and can be converted to mass change by Sauerbrey equation. The mass change that calculated from frequency shift of human albumin at 0.1 mg/ml was 114.1 ng or 1.03×10^{12} molecules of anti-human albumin on silver electrode surface. From this result, the combination of antibody-antigen at 0.05 mg/ml of anti-human albumin and 0.1 mg/ml of human albumin shown the interacted efficiency was 92% that correlated with the tendency to stable of the dose response curve. As well as, the detection limit that calculated from this curve was 9.5 ng/ml.

The previous study were presented the limit of detection to determined the microalbumin in many methods such as, Watts (53) shown the limit of detection to detected microalbumin in radio-immunoassay (1 µg/ml), radial immunodiffusion (2.5 µg/ml), immunoturbidimetry (2.5 µg/ml), and ELISA (6.25 ng/ml), Combiaso (56) shown the limit of detection to detected microalbumin in immunoassay by particle counting (0.4 µg/ml), Wayne (49) shown the limit of detection to detected microalbumin in radio-immunoassay (16 ng/ml), immunonephelometry (2 µg/ml), immunoturbidimetry (6 µg/ml), and HPLC (2 µg/ml), and Yang (107) shown the limit of detection to detected microalbumin in piezoelectric immunosensor (gold electrode) (4 ng/ml). From these data, the piezoelectric immunosensor with silver electrode can be comparable to those reference and routine methods.

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The study of binding capability of anti-human albumin on quartz crystal electrode surface after storage was continually operated. The F_c region of anti-human albumin was covalently linked with protein A that absorbed on the silver electrode surface thus the F_{ab} region of antibody can attach itself to target with suitability position. To evaluate the binding capability of anti-human albumin on quartz crystal electrode surface with protein A after storage, 14 groups of quartz crystals were assayed by protein A and anti-human albumin followed by the method above mentioned. All groups of quartz crystal were kept in moist chamber at cold room that control the temperature for 4-6^{o}C and each group was taken to applied human albumin in different time of storage. The result was shown in Figure 4.12. It was found that after 65 days of storage the binding capability of antibody stilled efficiently work. Then, in this study can kept quartz crystals that applied protein A and anti-human albumin in cold room not less than 65 days that has efficiently action in the next step of the experiment. However, this part must be continual study to reached the limitation of time to storage quartz crystals that applied protein A and anti-human albumin to detect the microalbumin.

According to all results above, the piezoelectric immunosensor that set for microalbumin look like can be used in the real situation. The study of clinical sample detection and comparison between piezoelectric immunosensor system and conventional system was latterly operated for confirm the hypothesis. After the calibration curve was generated and the limit of detection was evaluated, the clinical samples were detected individually with mass sensitive immunosensor and immunoturbidimetry (conventional method, Hitachi 917). The method comparison study must to trust method can be used for the comparison. Ideally would be reference method. Unfortunately, these may only be available at some place that allow handling the radio-material and has a financial support. Practically, the mew method has compared to an existing routine method. The microalbumin test is performed by the Hitachi 917 used for routine work in almost clinical laboratory. The result of comparative study of microalbumin detection in urine sample between mass sensitive immunosensor and conventional system is shown in Figure 4.13. The correlation curve shown good consistency between two methods ($R^2 = 0.9975$). The correlation analysis and paired samples t-test analysis were performed with SPSS, version 13, to evaluate

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the correlation and difference between two methods. The result of correlation analysis was shown in Table 4.1. The table shown a correlation value was 0.999 and significantly correlate ($P < 0.01$). That result described as good clinical comparability between two methods. As well as, the result of paired samples t-test analysis was shown in Table 4.2. The table shown a comparative value between two methods was not significantly different ($P > 0.01$). These data described as the results were not different between two methods. According the comparative results above, the piezoelectric immunosensor has shown high sensitivity, specificity and accuracy of the clinical samples test when compared with the conventional method (immunoturbidimetry). However, the trouble from a wide range of pH value may be interfered the combination of antibody-antigen. Then, the dilution of the clinical urine sample by PBS buffer pH 7.4 in appropriate dilution ratio was necessary to adjust the pH value of clinical samples to be the system range.

In Table 4.1, the results shown example values from calculated follow by the Sauerbrey equation to convert the frequency change into mass change and convert mass change into amount of molecule. From those results, the calculated value in each experiment was shown good system stability because of similar results.

In Figure 5.2, the figure shown schematic of this system by calculates the distance between close anti-human albumin. Calculate by surface area of silver electrode divide by the amount of antibody ($\approx 5.5 \times 10^{11}$ molecules). The computation was using area of one molecule of anti-human albumin that was about 2.28×10^{-13} cm^2. Thence, diameter of using area was calculated and subtract by the diameter of antibody to get the distance (≈24 Å).

In addition to high accuracy, sensitivity and specificity, the piezoelectric immunosensor also allows easy manipulated, label-free method that reduced safety concern about radioactive materials and keep away from interference of fluorescence method, and cheapness. The piezoelectric immunosensor could well analyzed in clinical samples in clinical laboratory and developed to an analytical method in the medical diagnostics conditions.

Nevertheless, the new method introduced into routine use must process to validate method to confirm that the analytical procedure operated for a specific test is suitable for its intended use. Results from method validation can be used to consider

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the reliability and consistency of analytical result. Method validation has obtained considerable attention in the many literature and many committees. J. Mark Green, 1996 (106), proposes the practical guide to analytical method validation that defines eight steps follow by:

- Specificity
- Accuracy
- Precision
- Limit of detection
- Limit of quantitation
- Linearity and range
- Robustness
- Stability

For this study, the specificity has done by use mixture of human albumin and immunoglobulin as a target. The result gave only signal from human albumin that shown the good specificity. The results and discuss of limit of detection, linearity and range has mention above. The results are shown in Table 4.1 demonstrated to satisfying stability in this method. Remainder, accuracy, precision, and robustness, must to suggesting evaluate for further study if introduce this method to routine use.

Moreover, the limitation of this system was the used long period of time for several steps of washing and drying that can be resolved by arranged quartz crystals that coated with protein A and anti-human albumin and kept in clod room before used. This resolution can be reduced the step of washing and drying to be one step but the system remained used a long time to drying before measured the frequency shift. The development of piezoelectric immunosensor to be a real-time system is necessary in the further study. The piezoelectric immunosensor that develop to be liquid phase detection by apply the flow system can be reach the system to be a real-time detection system. Therefore, the development of flow system must to suggesting for further study.

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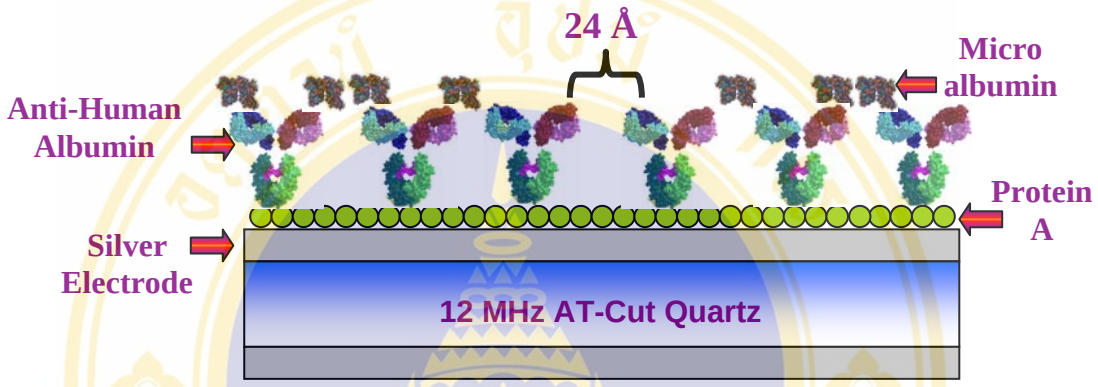

Figure 5.2 Schematic Diagram of Piezoelectric Immunosensor by Calculate

Protein A was coated on the silver electrode quartz surface. Next, anti-human albumin was immobilized; F_c of anti-human albumin binds to protein A and F_{ab} adheres specifically to microalbumin. The distance between close anti-human albumin was about 24 Å.

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CHAPTER VI
CONCLUSION

This piezoelectric immunosensor system was developed for effective detection of microalbumin in urine samples. The results of this study were concluded as following:

1. The optimal concentration and incubation time of protein A for anti-human albumin immobilization were 1.0 mg/ml and 2 hours at room temperature, respectively.
2. The optimal concentration and incubation time of anti-human albumin for microalbumin determination were 0.05 mg/ml and 2 hours at room temperature, respectively.
3. The estimated lower limit of detection from calibration curve was 9.5 ng/ml.
4. The effected from oxidation of silver surface gave insignificant irritation for this study.
5. Quartz crystals can be kept after applied protein A and anti-human albumin in cold room up to 65 days that has efficiently action in the combination with microalbumin.
6. The piezoelectric immunosensor suggested to good correlation with conventional method (immunoturbidimetry) to detect microalbumin in urine samples.
7. This immunosensor system performed high sensitivity for microalbumin detection in urine sample when compared with conventional method (immunoturbidimetry).

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APPENDIX

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No. MU 2007-066

Documentary Proof of Ethical Clearance
The Committee on Human Rights Related to
Human Experimentation
Mahidol University, Bangkok

Title of Project. Mass Sensitive Immunosensor for Microalbumin Determination
(Thesis for Master Degree)

Principle Investigator. Mr. Jittawat Sripratumporn

Name of Institution. Faculty of Medical Technology

Approved by the Committee on Human Rights Related to Human Experimentation

Signature of Chairman. .....
(Professor Dr.Srisin Khusmith)

Signature of Head of the Institute. (for) .....
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