CHAPTER 2 THEORIES AND LITERATURE REVIEW

This chapter contains two main parts which are the theories and literature reviews. The first part, theories, is divided into five sections which are acute kidney injury, biomarkers, immunoassay, the detection of NGAL and lateral flow immunoassay.

2.1 Acute kidney injury [1]

Kidneys are the organ in human body which has the important roles in the urinary system and also homeostatic functions. The kidneys' functions are to filter the excess substances from the body through urine and to reabsorb the necessary substances to control body's fluid balance.There are two types of kidney failure which are chronic kidney disease and acute kidney injury. Firstly, chronic kidney disease (CKD) or chronic renal disease is a loss in renal cell with progressive deterioration of glomerular filtration, tubular reabsorptive capacity, and endocrine functions of the kidneys over a period of months or years. It can be resulted from acute kidney injury. The operation, diabetes, and heart attack can cause chronic kidney disease. All forms of renal failure are indicated by a reduction in the Glomerular Filtration Rate (GFR). The last stage of the CKD is the loss of kidney function which the GFR is less than 5% of normal. Secondly, acute kidney injury (AKI) or acute renal failure (ARF) is rapid loss of kidney function which will increase blood levels of nitrogenous wastes and impair fluid and electrolyte balance. Normally, AKI is diagnosed in blood urea nitrogen (BUN) and creatinine. For the AKI patients, the concentration of BUN and creatinine is increased and the elimination rate of substances in urine is decreased when compared to normal people. This diagnosis still has the disadvantage which takes a long time at least 24 hours for the rising in creatinine level. AKI may also lead to metabolic acidosis, high potassium levels, uremia, changes in body fluid balance, and effects to other organ systems. The most common indicator of acute kidney injury isazotemia, which is an accumulation of nitrogenous wastes in the blood whilethe indicator of acute renal failure is decreasing in the GFR.

2.2 Biomarkers

Normally, serum creatinine and blood urea nitrogen are used for detection of AKI but they consume quite long time. Thus, the sensitive and specific biomarker is desired to indicate for AKI instead of serum creatinine and blood urea nitrogen. Biomarker, or biological marker, is in general a substance used as an indicator of a biological state.The alternative biomarkers for early AKI detection are kidney injury molecule-1 (KIM-1), interleukin-18 (IL18), N-Acetyl-β-D-glucosaminidase (NAG), cystatin C and neutrophil gelatinase associated lipocalin (NGAL).

2.2.1 Kidney injury molecule-1 (KIM-1) [2], [3], [4], [5]

KIM-1 is a type I cell membrane glycoprotein containing a novel six-cysteine immunoglobulin-like domain plus a threonine/serine and proline-rich domain

characteristic of mucin-like *O*-glycosylated proteins. It is undetectable in normal kidney tissue or urine, but is expressed at very high levels in the proximal tubule cell dedifferentiation from human kidney after ischemic renal injury. KIM-1 has been found to be an early biomarker for AKI because it is more sensitive, specific and stable for detection of AKI than serum creatinine and blood urea nitrogen in both cross-sectional and prospective adult clinical studies.

2.2.2 Interleukin-18 (IL18) [3], [4], [5],[6]

Interleukin 18 (IL-18) or interferon-gamma inducing factor is a proinflammatory cytokine that is induced and cleaved in the proximal tubule and subsequently can be easily detected in the urine following ischemic AKI in animal models. It is a 24 kDa inactive precursor which is splited by caspase-1 to generate its mature. Urinary IL-18 is an early, predictive and sequential AKI biomarker in children cardiac surgery patients. Urinary IL-18 levels displayed sensitivity and specificity for diagnosis of AKI, IL-18 concentration in patients is richer than IL-18 concentration in normal people. The result shows that IL-18 is significantly upregulated up to 48 hours prior to the increase in serum creatinine in patients.

2.2.3 N-Acetyl-β-D-glucosaminidase (NAG) [3], [4], [5]

N-Acetyl-β-D-glucosaminidase (NAG) is a lysosome enzyme that present in proximal tubular epithelial cells. NAG has a large size about 130 KDa, it prevents glomerular filtration. It is stable in urine across a range of pH and temperature. Renal disease and operation cause increased NAG in urine like serum creatinine. The measurement of quantitative NAG can use colorimetric and spectrophotometric methods. It has justified being a sensitive and strong indicator of AKI. However, urinary NAG levels are not constantly different between patients with or without AKI after cardiac surgery in adult.

2.2.4 CystatinC [3], [4], [5]

Cystatin C or cystatin 3 is a small cysteine proteinase inhibitor in tissues and body fluid which is synthesized and released into the blood at constant rate by all nucleated cells. Cystatin C is mainly used as a kidney biomarker. It has a small size about 13 KDa and positive charge at physiologic pH that makes it freely filtered at the glomerulus and reabsorbed by proximal tubule. Cystatin C is a better predictor than serum creatinine because it does not depend on age, gender, race, or muscle mass. The result of using cystatin C for prediction of AKI is detected within 12 hours after operation. Advantages of cystatin C are the commercial availability which provides results in minutes, routine clinical storage conditions and the etiology of AKI do not affect serum cystatin C measurements.

2.2.5 Neutrophil gelatinase associated lipocalin (NGAL) [3], [4], [5]

Neutrophil gelatinase associated lipocalin (NGAL) or lipocalin-2 (LCN2) is a protein of the lipocalin family which function is a growth factor. It is identified as a 25 KDa protein covalently bound to gelatinase from neutrophils. Normally, people have a low level of NGAL but the AKI patients have high NGAL concentration such as NGAL concentration has 10-fold in plasma and 100-fold in urine within 30 minutes then NGAL is easily detected in the blood and urine soon after AKI. NGAL is upregulated more than 10-fold within the first few hours after ischemic renal injury which earlier change than serum creatinine which used 1-3 days change. For many reasons, NGAL has been found to be a promising biomarker for early diagnosis, for predicting disease severity, and for predicting clinical outcomes. Therefore, plasma and urine NGAL is sensitive, specific, and highly predictive early biomarkers of AKI. In some studies, comparing the concentration after cardiac surgeries of NGAL and other biomarkers such as KIM-1 and NAG at the same time is indicated that NGAL is increased in few hours at highly level as it is shown in Figure 2.1. This result shows that NGAL is the early biomarker for AKI.

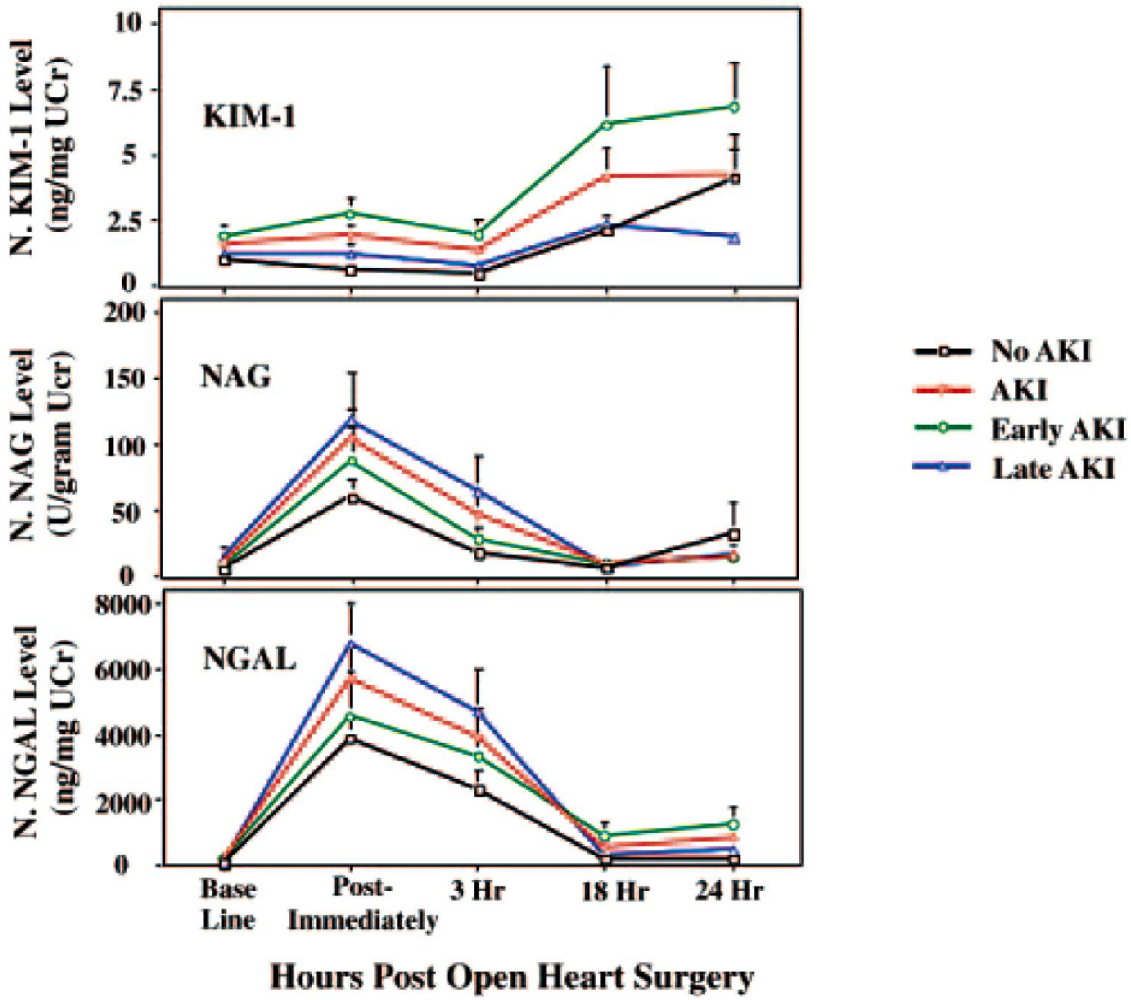

Figure 2.1 Pattern of urinary biomarker expression of KIM-1, NGA and NGAL at different time after cardiac surgeries. [7]

2.3. Immunoassay [8]

Generally, immunoassays are applied in medical and research purpose. Immunoassays are the biochemical test which is used to detect or quantify thesubstances such as serum, blood or urine (analyte) by using antibody or immunoglobulin. Normally, immunoassays are used for the analyte which presents at very low concentrations and cannot be determined by other measurements. Immunoassays are highly sensitive and specific. The antigen or the antibody for the other of the antibody or antigen pair can be used to accomplish immunoassay. The assay specificity depends on binding ability of antigen and antibody and elimination of other substances in the sample. Furthermore, the accuracy of the test is affected from highly affinity for the analyte which is depended on the individual assay format. The change in some physical characteristic is used for detection of the test. Detectable label is the most popular to detect the test which consists of radioactive elements used in radioimmunoassay, enzymes, fluorescent, chemiluminescent dyes, gold colloidal particles and etc.

There are four groups of immunoassays which are label free, reagent excess, reagent limited and ambient analyte. First is label free assay, an interaction between the antigen and antibody is generate to be molecular complexes which can be visible to the eyes. But in laboratory, the smaller amount can be detected and measured their ability to disperse a light. Thus, the concentration of a reagent antibody can be used to detect specific antigen and, likewise, a reagent antigen can be used to indicate specific antibody. If the reagent species is earlier coated onto cells or small particles, binding at much lower concentrations causes detectable precipitation or agglutination of the coated particles as shown in Figure 2.2.A and 2.2.B, respectively.

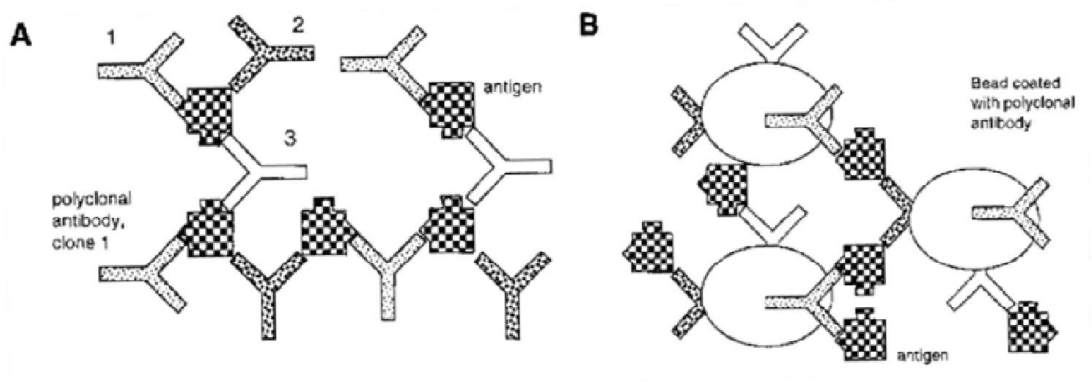

Figure 2.2 The label free assays (A) precipitated complexes (B) agglutinated complexes. [8]

Second, Reagent-excess assays are the assays that consume an excess concentration of labeled antigen or antibody to detect specific antibody or antigen, respectively. One-site reagent excess assays which proteins absorbed to nitrocellulose membrane are located by probing with labeled specific antibodies and immunostaining. For example, immunofluoresence assay which antigen in a tissue is visualized with specific antibodies conjugated to fluorescein as shown in Figure 2.3.C. Otherwise, two-site

assays or sandwich assays are the assays which have separate binding reactions specific for two sides on the analyte as shown in Figure 2.3.D. In the same way, two-site assays are used to detect specific antibodies of a particular immunoglobulin class or subclass.

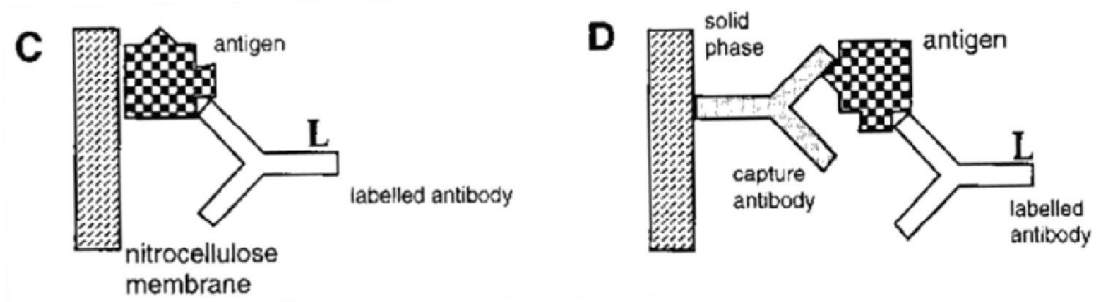

Figure 2.3 The reagent excess assays (C) one site reagent assay (D) two sites reagent assay. [8]

Thirdly, Reagent-limited assays or competitive assays which may be labeled antigen or antibody are used to measure concentrations of large antigen and small analyte as shown in Figure 2.4.E and 2.4.F. In contrast to other immunoassays, the immune complexes measure or detected do not contain analyte; thus, all assays will give inverse standard curves when concentration of bound label is plotted against analyte concentration. Reagent-limited assays are normally less sensitive than reagent excess assay; moreover, they are difficult to develop and optimize, and are not often developed for small-scale laboratory applications.

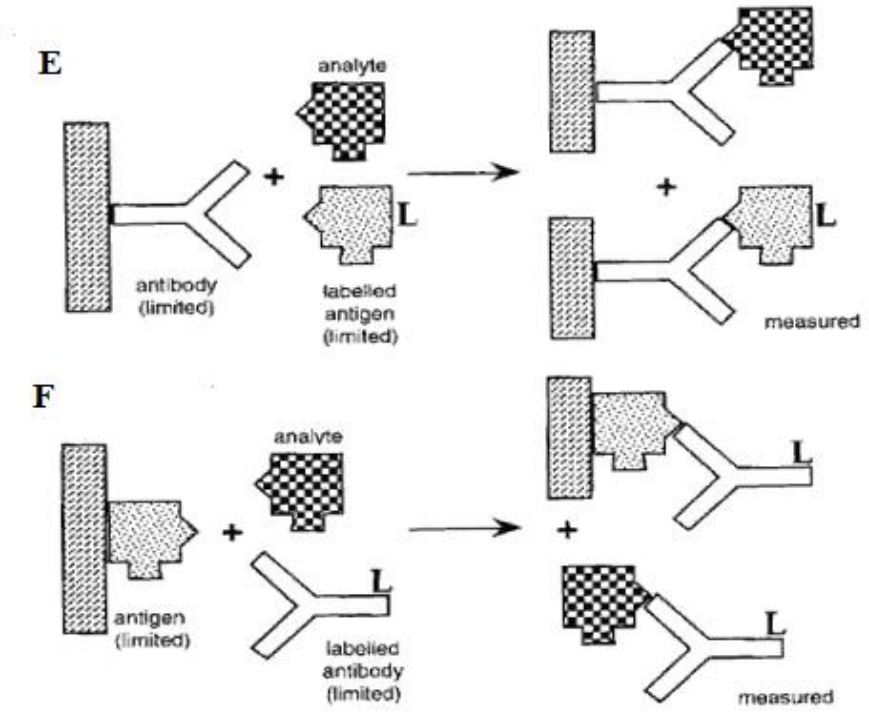

Figure 2.4 The reagent limited assay (E) labeled antigen (F) labeled antibody. [8]

And lastly is ambient analyte immunoassays, they are used to perform the low concentration of the capture antibodies. Their presence does not affect the concentration

of free analyte in the reaction medium.To make it possible such as a very specific activity system, fluorescent labels are used, and the small amount of capture ligand is coated at very high density within a microspot.

2.4. The methods of NGAL detection

There are several methods available to detect NGAL, such as ELISA (enzyme-linked-immunosorbent assay), ARCHITECT, Triage, and lateral flow immunoassay.

2.4.1 ELISA [9], [10], [11]

ELISA stands for enzyme-linked-immunosorbent assay. ELISA is a plated-based assay which is used to detect and qualify substances such as proteins, antibodies and hormones. ELISA has been used as diagnostic equipment in medicine and plant pathology. In this method, an antigen must be immobilized to a solid surface and then antibody which is linked to an enzyme is added to bind complexly with the antigen. After that substance is added to be incubated with the enzyme. When the reaction is complete, the color is presented. The amount of color produced is proportional to the amount of primary antigen in the substance. ELISA is typically performed in 96-well (or 384-well) plates which will passively bind antibodies and proteins as shown in Figure 2.5.

Figure 2.5 The ELISA plates [10].

The main ELISA formats can be divided into four formats which are direct ELISA, indirect ELISA, sandwich ELISA, and competitive ELISA. First is direct ELISA. In this method, an antigen is coated on the plate and an antibody is directly conjugated to an enzyme which is used for detection as shown in Figure 2.6.A. The main advantages of direct ELISA is it gives faster results with less error. Second, indirect ELISA is the easiest and most popular format of ELISA. An antigen coated to plate is detected in two stages or layers which are firstly; unlabeled primary antibody (specific for antigen) is applied and secondly; an enzyme-labeled secondary antibody (an anti-species antibody)

is bound to the first antibody as shown in Figure 2.6.B. This method has many advantages such as increased sensitivity, cost savings and flexibility. Next is sandwich ELISA, it typically requires the use of matched antibody pairs. The first antibody is coated to the plate and them the sample is added to the well. A second antibody layer, the detection antibody, follows this step in order to measure the concentration of the analyte. If the detection antibody is conjugated to an enzyme, then the assay is called a direct sandwich ELISA as shown in Figure 2.6.C. If the detection antibody is unlabeled, then a second detection antibody will be needed resulting in an indirect sandwich ELISA as shown in Figure 2.6.D. This method is high specificity, suitable for complex samples, and flexibility and sensitivity. And the last one is competitive ELISA. This is the most complex ELISA and is used to measure the concentration of an antigen (or antibody) in a sample by observing interference in an expected signal output. This method can be based upon any of the above ELISA formats. It is most often used when only one antibody is available to the antigen of interest or when the analyte is small.

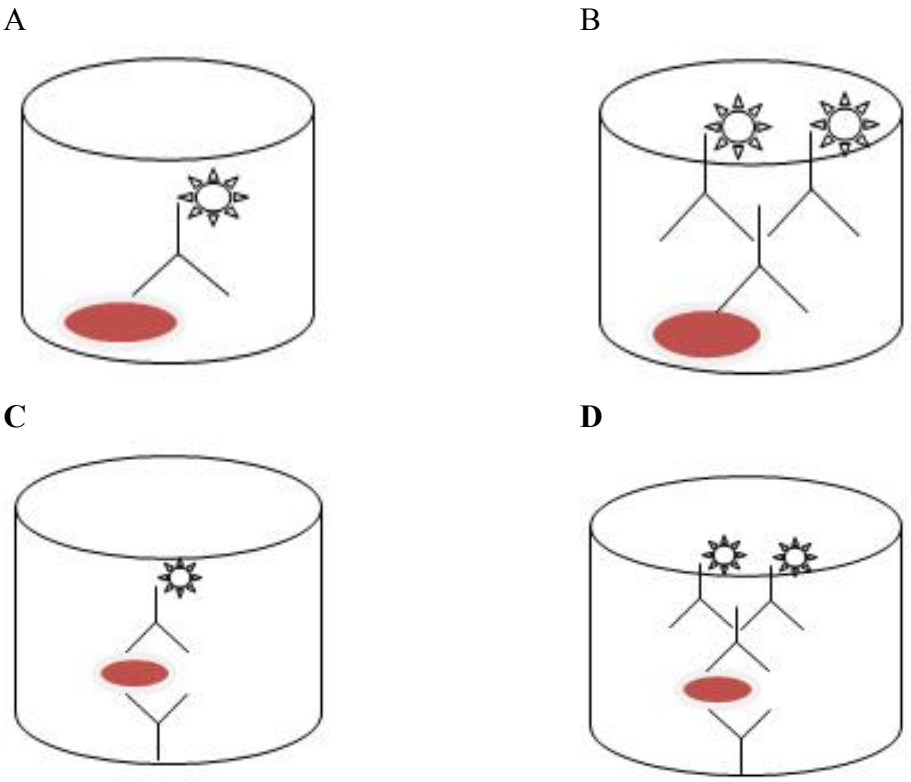

Figure 2.6 The formats of ELISA (A) Direct ELISA (B) Indirect ELISA (C) Direct sandwich ELISA and (D) Indirect sandwich ELISA. [10]

2.4.2 Architect [12], [13], [14]

ARCHITECT urine NGAL assay is a non-competitive two-site sandwich assay using chemiluminescent microparticle immunoassay technology for the quantitative detection of NGAL in urine. It requires only 150 µl of urine for measurement. This method is easy to perform without pretreatment and then the result will be presented within 35 minutes with high accuracy (99%) when compared with ELISA. However, this method requires specific equipment which is very expensive. Furthermore, the asset has a dynamic range that extends to 1500 ng/ml. Controls are available with the following

concentrations: 20 ng/ml (low), 200 ng/ml (medium) and 1200 ng/mL (high). The sensitivity is 0.82 and the specificity is 0.90 for prediction of AKI using a cut-off value of 100 ng/ml. Thus, the ARCHITECT platform is used to detect urine NGAL with the accurate results.

2.4.3 Triage [12], [15]

Triage assay is a point of care kit which has been developed for the clinical measurement of NGAL in plasma. This device is a rapid point-of-care fluorescence immunoassay which is used with the Triage meters. It requires only 250 µl of blood for measurement and then the result is approximately shown within 15 minutes with 94% accuracy when compared with ELISA, but the precision of this method is lower than ARCHITECT. However, there are a lot of advantages which are no sample processing, no precision pipetting, or no daily liquid controls.

2.4.4 Lateral flow immunoassay (LFIA) [16]

Lateral flow immunoassay (LFIA) was derived from the latex agglutination assay. The main application of this assay was the human pregnancy test which used urine testing for medical diagnosis. This method has many advantages which are easy operation, simple to scale up for high production, stable and high sensitivity and specificity; however, it still has some problems such as miniaturization of sample volume which requires below microliter level.

There are two formats of a lateral flow immunoassay which are direct (sandwich) and competitive (inhibition) assay. Direct assay is usually used for larger analyte with multiple antigenic. The positive result is indicated by the presence of the test line. If the analyte is less than the desire, some of the conjugated particles will not be captured at the test line then the negative result is presented as shown in Figure 2.7.

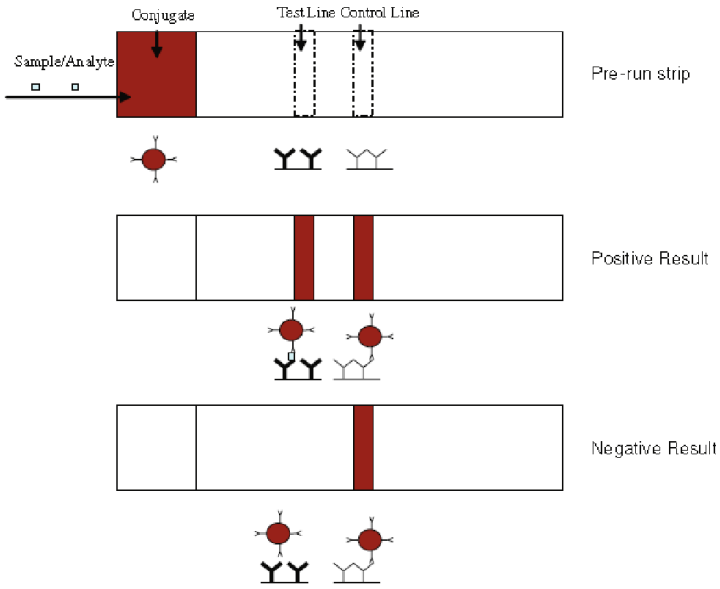

Figure 2.7 Direct solid-phase immunoassay. [16]

Competitive assay is used for small molecules with single antigenic determinants which cannot bind to two antibodies at the same time. The positive result is indicated by the absence of a test line. In contrast, negative result will show both test line and control line. This format has reverse method of indicating result from direct assay as shown in Figure 2.8.

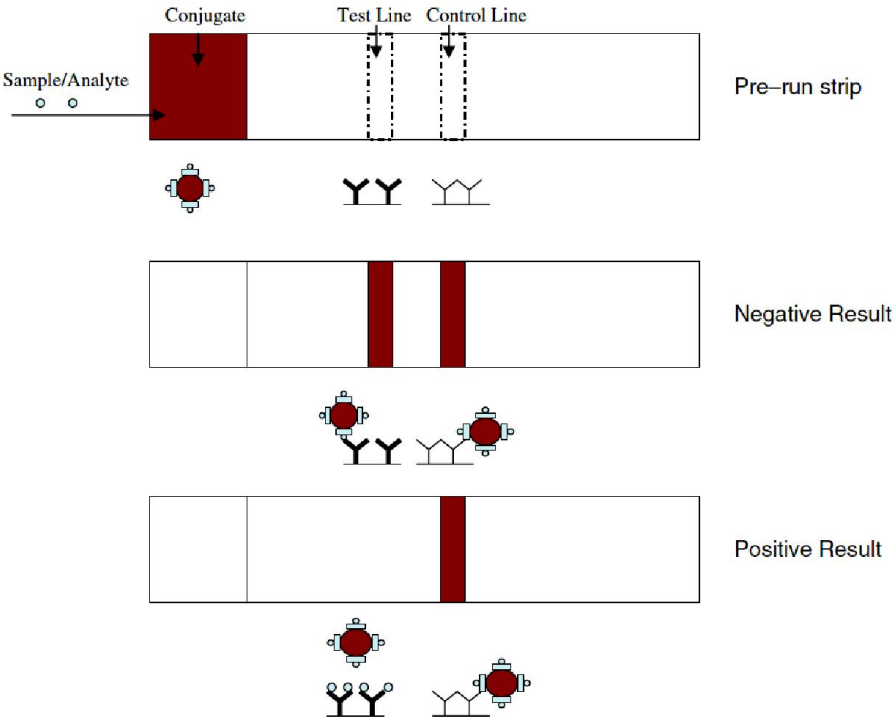

Figure 2.8 Competitive assay. [16]

The typical configuration of a lateral flow immunoassay is shown in Figure 2.9. It consists of variety of materials which are sample pad, conjugate pad, nitrocellulose membrane and absorbent pad (wick) and backing card. All materials overlap onto one another and are mounted on a backing card using pressure-sensitive adhesive. When a test is run, sample is added to the sample pad which is the proximal end of the strip. The sample is treated to make it compatible with the rest of the test, and then it migrates to the conjugate pad where a particle conjugate has been immobilized. Normally, the particle can be use colloidal gold, or a colored, fluorescent paramagnetic monodisperse latex particle. The particle has been conjugated to antibody or antigen depending on the assay. After that sample re-mobilizes the dried conjugate, the analyte in the sample interacts with the conjugate and then migrates into the next section which is reaction matrix. The reaction matrix is a porous membrane, onto the other specific biological component of the assay has been immobilized such as protein, antibody and antigen. It is laid in down bands in specific areas to create test and control line. Excess reagents move past the capture lines and are entrapped in the wick. The assay formats can be either direct or competitive.

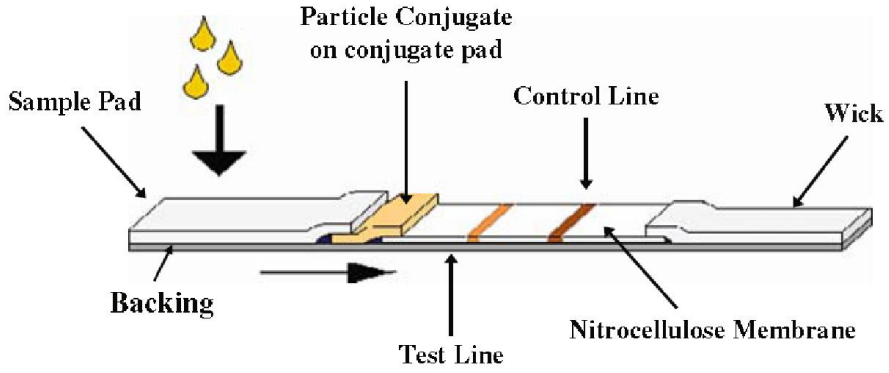

Figure 2.9 Typical shape of a lateral flow immunoassay test strip. [16]

2.5 Carbon nanoparticles [21], [22], [23], [24]

Carbon nanoparticles (CNP) are an inert chemical in term of surface charge and no oxidation, usually ranging from 1-100 nm in one dimension. The applications of CNP are used in pharmacy, drug delivery and many industrial and commercial practices. CNP is presently the one alternative of several labels to use as a label for lateral flow immunoassay. It is the cheapest labels, more sensitive, very stable suspension, and easy to perform. Moreover, CPN can be adhered with a range of biomolecules such as DNA, antibodies, and protein and the resulting conjugates can be used to detect analytes of high or low molecular mass.

The preparation of colloidal carbon nanoparticle conjugate with proteins is easy to perform and very stable. Colloidal carbon conjugate is stable after preparation at least 1 year at 4℃ but it might tend to aggregation. However, it was dissociated by ultrasonic vibration (G. J. van Dam et al., 2004). Even though, the conjugates were kept for long time, they were still shown the similar results as the initial experiments (Van Amerongen et al., 1993).

Noguera et al. [22] studied carbon nanopaticles in lateral flow method to detect and identify the different Shiga toxinproducing *Escherichia coli* virulence factors. The results of this study showed that the method development is reliable, cost-effective, easy to use and fast; moreover, the results are achieved within 40 min.

Tang et al. [23] studied sandwich-type conductometric immunoassay of alpha-fetoprotein in human serum using carbon nanoparticle as labels. The detection antibodies were prepared using nanocarbon-conjugated horseradish peroxidase-labeled anti-AFP. This study was compared to the conventional sandwich-type immunoassay, the presence of carbon nanoparticles could obviously improve the sensitivity of immunosensor that exhibited good dispersion in the solution.

2.6 Dot blot test [25]

A dot blot is a technique in molecular biology used to detect biomolecules, and for detecting, analyzing and identifying proteins. This method is a method of applying proteins directly onto a membrane. A dissolved sample is pulled through the membrane by either applying a vacuum, absorption or intrusion; proteins bind to the membrane and the other sample components pass through. The proteins on the membrane are then available for analysis. This technique can be used either as a qualitative method for rapid screening of a large number of samples or as a quantitative technique. It is especially useful for testing the suitability of experimental design parameters.

2.7 Previous works

Jaksunimitr C. studied the development of a lateral flow immunoassay test strip for the detection of NGAL protein. The test strip used the same technology as the home pregnancy test. Gold nanoparticles were used as a label in this study.The cut-off values of NGAL concentrations in PBS and urine samples were 110 ng/ml and 90 ng/ml, respectively. On the other hand, the color intensity of test line was too pale for clear result. An increase in the amount of antibody on the gold conjugate lowered the cut-off value but did not increase the color intensity at the test line.

Kulcharoen K. developed a lateral flow immunoassay test strip for the detection of NGAL protein. Due to the previous work, thecolor intensity of test line was too pale for clear result. In this study, the test strip was still the same format as the previous study and gold nanoparticles were used as a label. The researcher added 30 µL of silver enhance kit onto the test line, the band intensity could be increased by 5.82% of the band intensity before the treatment. Moreover, the researcher added 3% of methanol onto the test line could increase the band intensity to 14.44%.But adding both 3% of methanol and silver enhancer kit onto the test line, it could increase the band intensity up to 24.76%. Therefore, adding both 3% of methanol and silver enhancer kit onto the test line was the best way in this study to improve the color intensity of test line. However, the color intensity of the test line was still pale at concentrations lower than 500 ng/mL.

Nuanla-ong N. developed a lateral flow immunoassay test strip for the detection of NGAL protein. Due to the previous work, the color intensity of test line was too pale for clear result. In this study, the test strip was still the same format as the previous study but carbon nanoparticles were used as a label instead of gold nanoparticles.The color intensity of the test line was clearly visible at 500 and 1,000 ng/ml of NGAL protein. Moreover, the cut-off concentration of carbon label test strips was compared to that of the gold label test strip. The result showed that the cut-off concentrations of the carbon label test strips and gold label test strips were 200 ng/ml and 500 ng/ml, respectively.
The carbon label test strips gave darker color intensities of the control lines and the test lines, making it easier for users to analyze the results visually.