2. วิธีดำเนินการวิจัย (Materials and Methods)

2.1 Gold Nanoparticle Synthesis and characterization

- Preparation of gold nanoparticles

Gold nanoparticles (AuNPs) that was used throughout the project is citrate-reduced gold nanoparticles with concentration equal to 100 ppm when calculated based on amount of $HAuCl_4$ (equivalent to 50 ppm when calculated based on amount of Au^0). From previous study, spherical-shape gold nanoparticles with size approximately 10 nm in diameter were the most appropriate characteristics to be used in biosensing technology. Hence, we chose the modified Turkevich *et al.* method to be used in gold nanoparticles preparation due to the fact that gold nanoparticles from the method would meet the conditions stated above.

- Reagents
  1. 12.7 mM tetrachloroauric acid ($HAuCl4.3H_2O$)
     - ➢ Dilute 1.5 mL of 1% w/v $HAuCl_4.3H_2O$ (prepared from gold (III) chloride trihydrate (Sigma, cat. No. G4022)) in 1.5 mL of DI H2O
  2. 38.8 mM trisodium citrate ($C_6H_5Na_3O_7.2H_2O$)
     - ➢ Dissolve 34.2 mg of trisodium citrate (Merck, cat. No. A661048) in 3 mL of DI H_2O
  3. Deionized water (DI H_2O)

Figure 2.1 - 12.7 mM tetrachloroauric acid

Figure 1.2 - 38.8 mM trisodium citrate

- Equipments
  1. 50-mL glass flask
  2. 100-mL glass flask
  3. Hot plate with magnetic stirring function
  4. Reflux column
  5. Magnetic stirrer bar
  6. 1-mL auto-pipette and pipette tips

<u>Synthesis procedure</u>

Step 1 – Add 2 mL of 12.7 mM tetrachloroauric acid solution into 49 mL of DI H_2O in clean 50-mL glass flask with magnetic stirrer bar. Place the apparatus on hot plate and boil while stirring.

Step 2 – Add 1.84 mL of 38.8 mM trisodium citrate solution to 49 mL of DI H_2O in clean 100-mL glass flask with magnetic stirrer bar. Set reflux column on the flask will hot plate temperature controller on top of the flask. Put the intact apparatus on hot place and boil while stirring.

Step 3 – When the solutions in both flasks are boiled, slowly pour the diluted tetrachloroauric acid solution into diluted trisodium citrate solution. Reaffirm that the reflux column is firmly located and set the controlled temperature to 70°C. Stir vigorously.

Step 4 – Remain the controlled temperature until red-coral colored solution of gold nanoparticles is acquired. Then, continue to boil for 5 minutes and let it to cool down to room temperature. Leave the solution overnight while stirring.

Step 5 – Disassemble the apparatus and transfer gold nanoparticles colloid to a clean covered glass container and keep away from light.

Step 6 – Some of gold nanoparticles colloid are characterized by UV-visible spectrophotometer, nanosizer, and transmission electron microscope (TEM).

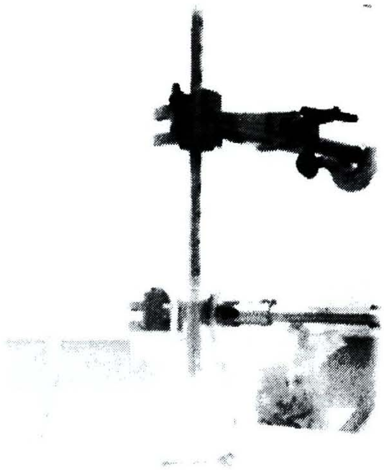


Figure 2.3 - Diluted tetrachloroauric acid during boiling on the hot plate

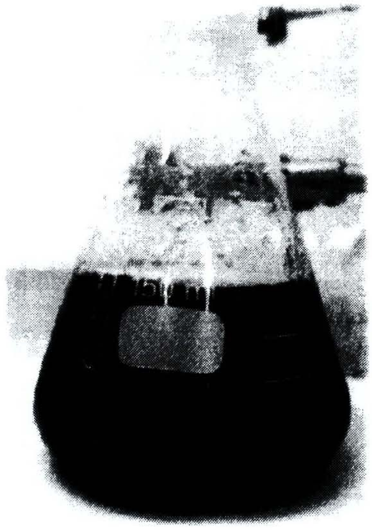


Figure 2.4 - Final 100 ppm gold nanoparticles colloid with a clear orange-red color

Figure 2.5 : UV/Vis spectrophotometer

2.2 Evaluation of in vitro gold nanoparticle cytotoxicity

2.2.1 HeLa cell culture

This study used HeLa cell lines (cervical cancer cells) which were kindly provided by Prof. Dr. Apiwat Mutirangura. See figure 10 for the morphology of HeLa cells. HeLa cell lines were maintained in DMEM medium, supplemented with 10% fetal bovine serum and 1% antibiotics. Cells were incubated at 37 °C with 5% CO_2 atmosphere. The cells were subcultured and fed as needed. For the experiment, cells were seeded in 24 well plate at a density of 1×10^5 cells per well in 500 µL of complete medium, triplicate samples. The cells were incubated for 24 hours at 37 °C with 5 % CO_2. After 24 hours incubation, the cells were added with DMSO as positive control and various concentrations gold nanoparticles (10, 50, and 100 ug/mL respectively). After that, cells were further incubated for 1, 2, and 3 days at 37 °C with 5 % CO_2.

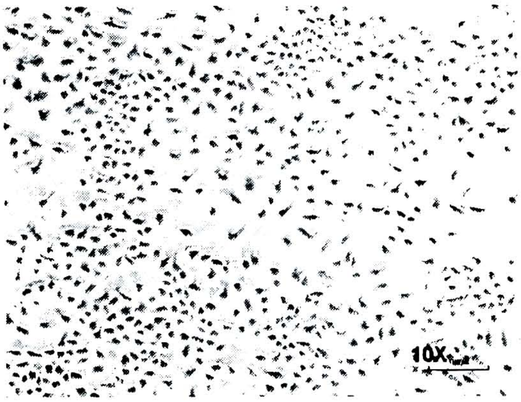


Figure 2.6 The morphology of HeLa cells.

2.2.2 Determination of cell morphology by Light Microscope (LM)

The effect of gold nanoparticles on cell morphology was determined using light microscope. After incubation cells with gold nanoparticles, cells were monitored and taken the picture by light microscope. The morphology of gold nanoparticles treated groups was compared with negative and positive control.

2.2.3 Determination of intracellular morphology and fate of gold nanoparticles by Transmission Electron Microscope (TEM)

The effect of gold nanoparticles on intracellular morphology as well as gold nanoparticles trafficking was determined using Transmission Electron Microscope (TEM). Cells were prepared as follows: after incubation with gold nanoparticles, cells were trypsinized and washed with PBS 2 times (10 minutes), followed by centrifugation at 2000 rpm for 5 minutes. The precipitate was prefixed in 2% glutaraldehyde for 2 hours and washed with PBS 3 times (10 minutes). Then, the precipitate was postfixed in 2% osmium tetraoxide for 1 hour, centrifuged at 14000 rpm for 5 minutes and washed with PBS 1 time (10 minutes). After that, the precipitate was dehydrated in ethanol series (50%, 70%, 80%, 95%-2 times and 100%-3 times, respectively). The precipitate was infiltrated in 100% propylene oxide for 2 hours, propylene oxide: epoxy resin (1: 1) for 3 hours and 100% epoxy resin for 18 hours. Finally, the precipitate was embedded in epoxy resin at 60°C overnight. Epoxy resin blocks were cut as ultra thin section (~ 60-90 nm), laid on the grid and stained with uranyl acetate and lead citrate. Samples were taken to monitor and take pictures by transmission electron microscope. The intracellular morphology of gold nanoparticles treated groups were compared with control groups.

2.2.4 *In vitro* Cytotoxicity assay (MTT)

In vitro Cytotoxicity was determined by MTT assay (3-(4,5-dimethylthia zol-2-yl)-2,5-diphenyl tetrazolium bromide). MTT assay is a colorimetric assay according to a tetrazolium-based compound is reduced to formazan by mitochondrial dehydrogenase in the living cells. Whereas, dead cells can not reduce tetrazolium-based compound to formazan because of lacking of mitochondrial dehydrogenase. The amount of formazan is directly proportional to the number of living cells in the culture. Formazan was measured with a spectrophotometer at 570 nm.

For the experiment, cells were seeded in 24 well plate at a density of 1×10^5 cells per well in 500 µl of complete medium, triplicate samples. The cells were incubated for 24 hours at 37 °C with 5 % CO_2. After 24 hours incubation, cells were exposed to various concentrations of gold nanoparticles. Then, cells were further incubated for 1, 2 and 3 days. After 1, 2 and 3 days incubation, cells were washed with PBS 2 times, followed by added 350 µL of the tetrazolium-based compound to each well. Cells were incubated in the incubator for 30 minutes. Cells were removed the tetrazolium-based compound and dissolved formazan with 1 ml of DMSO: glycine buffer (9: 1). The formazan solution was pipetted into cuvettes and measured optical density by spectrophotometer at 570 nm. The relative cell viability (%) relates to control wells containing cell culture without gold nanoparticles was calculated by using the following equation.

$$[A]_{test} / [A]_{control} \times 100$$

Where $[A]_{test}$ is the absorbance of the test sample and $[A]_{control}$ is the absorbance of control sample(39).

2.3 Physiochemical Effects of DNA-coated goldnanoparticles

2.3.1 Preparation of DNAs

All oligonucleotides were prepared by diluting a 200 µM oligonuclotide stock solution to 20 µM. The oligonucleotide sequences used in this experiment are shown in Table 2.3.1.

Table 2.3.1 Oligonucleotide sequences

Oligonucleotides	**Sequence (5' to 3')**
Oligo1 (target leptospirosis)	TTTATTCGTCGTTACACTAC
Oligo2 (leptospirosis probe)	GTAGTGTAACGACGAATAAA
Oligo3 (non-complementary to target)	CCGAGCAATAAATCTTTACC
Oligo4 (1 mismatched complementary to target)	CTAGTGTAACGACGAATAAA

A stock DNA was prepared by adding an oligonucleotide into the hybridization buffer (0.2xSSC containing 5% formamide and 1% dextran sulfate) at room temperature for 30 min prior to use. Ratios of the oligonucleotide and the hybridization buffer are shown in table 3-2.

Table 2.3.2 DNA mixture composition (Oligo1 : Oligo2 : Oligo3 : Oligo4 : hybridization buffer)

DNA mixture	Oligo1 (μL)	Oligo2 (μL)	Oligo3 (μL)	Oligo4 (μL)	Hybridization buffer (μL)
mixture1 (ssDNA)	6	-	-	-	2
mixture2 (ssDNA)	-	6	-	-	2

mixture3 (dsDNA)	3	3	-	-	2
mixture4 (ssDNA)	3	-	3	-	2
mixture5 (1 mismatched dsDNA)	3	-	-	3	2

2.3.2 Aggregation of gold nanoparticles

2.3.2.1 Effect of buffer on aggregation

Into 5 eppendorf tubes contrary, 250 µl of 100 ppm AuNPs, 10, 50, 100, 150 and 200 µl of hybridization buffer were added. The solutions were *occasionally shaken* and allowed to stand for at least 30 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 µl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

2.3.2.2 Effect of salt on aggregation

Into 5 eppendorf tubes contrary, 250 µl of 100 ppm AuNPs, 1, 2, 3, 4, and 5 µl of 2M NaCl were added. The solutions were *occasionally shaken* and allowed to stand for at least 30 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 µl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

2.3.2.3 Effect of DNA on aggregation

Into 15 eppendorf tubes contrary, 250 µl of 100 ppm AuNPs, 0.1, 0.2, 0.3, 0.4 and 0.5 µl of mixture1-3 were added. Subsequently, 4 µl of 2M NaCl was added. The solutions were *occasionally shaken* and allowed to stand for at least 30 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 µl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

2.3.2.4 Colorimetric detection of non-complementary DNA, complementary DNA and 1-mismatched complementary DNA

Into 3 eppendorf tubes contrary, 250 µl of 100 ppm AuNPs, 0.3 µl of mixture3-5 were added. Subsequently, 4 µl of 2M NaCl was added. The solutions were *occasionally shaken* and allowed to stand for at least 30 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 µl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

2.4 Fabrication of Lateral Flow test Strip

The basic principle of AuNPs-based NALF detection system involves the design of thiol-modified specific oligonucleotide probes on AuNPs. If there are complementary targets that can form sandwich hybridization with both thiolated probes on AuNPs and another target-specific probes on test line of the NALF test strip, the functionalized-AuNPs will accumulate at the test line revealing a distinct red color. Previous study reported the utilization of NALF to detect human genomic DNA samples without PCR amplification. Their research group found that the AuNPs based-NALF assay on a test strip had enabled naked eye detection of the target DNA with a very high sensitivity.

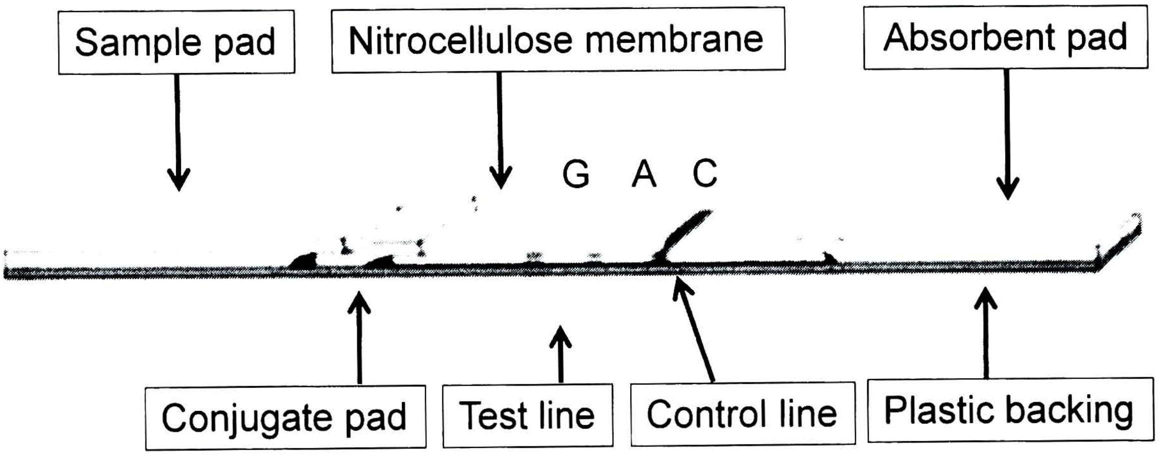


Figure 2.7 Model of components of Lateral flow strip test

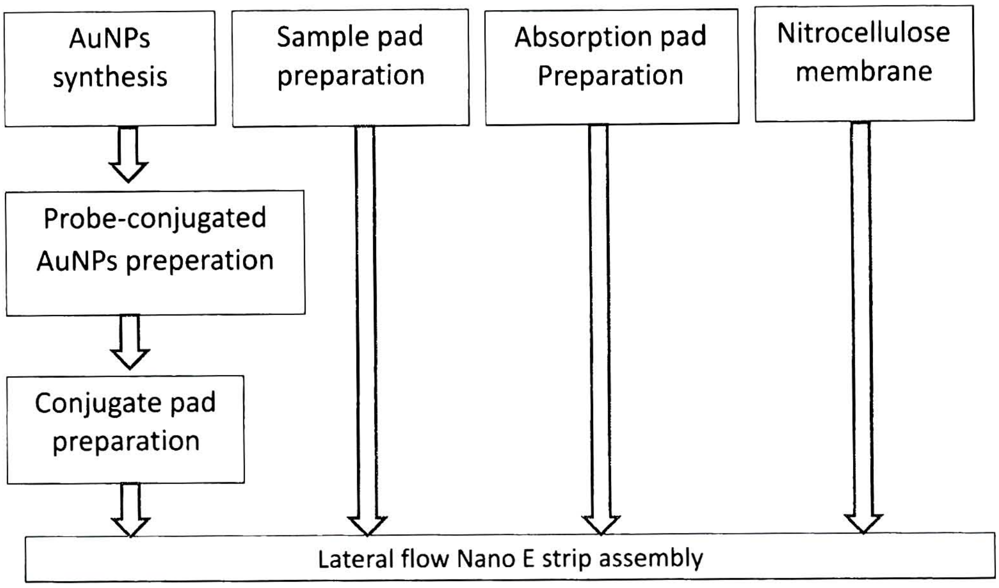


Figure 2.8 Processes of Lateral flow strip test assembly

2.5 Optimization of influencing parameters for fabrication of gold nanoparticle-based nucleic acid lateral flow strip test

2.5.1. Chemical used

- DL-Dithiothreitol (DTT, Sigma-Aldrich, Canada)
- Tris (2-carboxyethyl) phosphine (TCEP, Sigma-Aldrich, USA)
- Hydrogen tetracholoaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$, Sigma-Aldrich, USA)
- Trisodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$, Merck, Germany)
- NAP-5 columns (GE Heathcare, UK)
- Glass microfiber (Ahlstrom; grade 8964, USA)
- Absorbent pad (Ahlstrom; grade 319, USA)
- Nitrocellulose membrane (Millipore; Hiflow plus HFB24004, Millipore, UK)
- Milli-Q water (> 18 MΩ, Labconco : Water Pro PS, USA)

2.5.2 Materials

The experimental probe sequences were 5' GGT AAA GAT TTA TTG CTC GGT TTT TTT TTT- (CH2)3-(Thiol) 3' [probe#1 : detection probes], 5' GCA CTT GGT GTA GCA ATT AAT GCT G-(CH2)3- (Thiol) 3' [probe#2 : detection probes], 5' (biotin)-GGC CAA GTT AAA CTC TAT GCT GAC 3'[probe#3 : capture probes on the test line] and 5' CAG CAT TAA TTG CTA CAC CAA GTG C-(biotin) 3' [probe#4 : complementary sequence with probe#2 on the control line]. Synthetic target sequence was 5' AGC AGC ATT AAT TGC TAC ACC AAG TGC TCC TTA AGT CAG CAT AGA GTT TAA CTT GGC CGA 3'. All reagents were prepared with Milli-Q water (> 18 MΩ).

2.5.3 Gold nanoparticles synthesis

Gold nanoparticles (AuNPs) were synthesized by adding 38.8 mM trisodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in boiling 0.02%w/v hydrogen tetracholoaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$) in Milli Q water with vigorous stirring. The solution turned deep-red within 2 min after which it was boiled for additional 15 min and then stored at $4^{O}C$ away from light. The AuNPs were characterized by UV/Vis spectroscopy (Beckman coulter DU ® 800; maximum absorption at 520 nm) and transmission electron microscope (TEM; HITACHI Model H-7650 : 100 kV). Their absorption spectrum and TEM micrograph are shown in Fig.1A and Fig.1B respectively.

2.5.4 Conjugation of thiolated probes on AuNPs

DL-dithiothreitol (DTT) and tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were used separately as a sulfhydryl reductant to reduce the disulfide linkages of the probes to the thiol forms before conjugated to AuNPs. The conjugation was done in a conventional way as described in previously reported protocols with a slight modification. Briefly, 100 μM probe#1 (supplied form) were reduced with DTT. The probes were then purified with a NAP-5 column before added to AuNPs and finally incubated overnight under a gentle shaking. After the incubation, the shaking was continued while a salting buffer was periodically added every 8 hours to obtain a final NaCl concentration of 0.7 M. After the first and the last 3 times of the salting additions, the conjugates were sonicated for 10 sec. The final solution was centrifuged at 12,000 rpm for 15 min and the sediment (AuNPs-probes) was washed with a washing buffer twice. The supernatants (excess thiolated probes) from all three centrifugations were analyzed under UV/Vis spectroscopy and calculated for

% probe loss = [OD_{260nm} x 33μl/ng x Volumn (μl)]/ initial thiolated probes (ng) x 100).

The functionalized AuNPs were stored at $4^{O}C$. For the TCEP treatment, the procedures were similar to the DTT protocol as mentioned above. TCEP was added up to a final concentration of 0.1 mM and no column purification needed.

2.6 Preparation, modification, and testing of antibody-conjugated AuNPs

Gold nanoparticles were attached by antibody which has ability to identify and specifically bind with antigen. After attachment, the nanoparticles received capability to locate themselves onto the place where specific antigen was presenting. Then, when many of gold nanoparticles were biologically gathered together in close space (*e.g.* around antigen protein on nitrocellulose membrane), the nanoparticles did spontaneously aggregate and solidify – which will appear as a red/black dot on the membrane.

Our project has focused on developing gold nanoparticles to detect Leptospirosis or Weil's disease. Major antibody which had been used throughout the project was polyclonal antibody of *Leptospira pomona* in laboratory grade. However, a set of antibodies, which included monoclonal and polyclonal antibody of *Leptospira pyrogenase*, were borrowed from microbiology laboratory in faculty of medicine. Meanwhile, set of antigens that had been employed during this project were agitated bacterial cell fracture taken from bacterial cell culture plates. List of available antigens were *Leptospira pomona*, *Leptospira pyrogenase*, *Staphylococcus aureus*, and *Escherichia coli* while the blank solution was 1X Phosphate Buffer Saline or PBS.

Many conditions and factors were adapted and adjusted to get the most optimistic sensitivity and specificity of antibody-conjugated gold nanoparticles when compared to conventional detection method. The conditions that had been modified did take place in many of different step depended on pertinence.

- Reagents
  1. 100 ppm gold nanoparticles (AuNPs) from the first part of this project
  2. 0.5 mg/mL polyclonal antibody of *Leptospira pomona* as stock antibody
  3. Blended cell fractures of *Leptospira pomona*
     - ➢ Agitate 10^8 cells of *Leptospira pomona* bacteria in 1 mL of sterile DI H_2O by sonicator
  4. Blended cell fractures of *Leptospira pyrogenase*
     - ➢ Agitate 10^8 cells of *Leptospira pyrogenase* bacteria in 1 mL of sterile DI H_2O by sonicator

5. Blended cell fractures of *Staphylococcus aureus*
    - ➢ Agitate 10^8 cells of *Staphylococcus aureus* bacteria in 1 mL of sterile DI H_2O by sonicator
6. Blended cell fractures of *Escherichia coli*
    - ➢ Agitate 10^8 cells of *Escherichia coli* bacteria in 1 mL of sterile DI H_2O by sonicator
7. 1X Phosphate Buffer Saline (PBS)
    - ➢ Prepared from protocol in Appendix A1.7 of Sambrook and Russell, *Molecular Cloning; A laboratory manual*, 3rd edition, Volume 3.
8. 0.4 M sodium carbonate (K_2CO_3)
    - ➢ Dissolve 2.76 g of Potassium Carbonate (Sigma, cat. No. P1472) in 50 mL of DI H2O.
9. 2% polyethylene glycol in 1X PBS (PEG in PBS)
    - ➢ Dissolve 10 g of polyethylene glycol (Sigma, cat. No. P2263) in 500 mL of 1X PBS.
10. 10% w/v bovine serum albumin (BSA) in 1X PBS
    - ➢ Dissolve 5 g of albumin from bovine serum (Sigma, cat. No. A4503) in 50 mL of 1X PBS.
11. 10% w/v dairy milk in 1X PBS
    - ➢ Dissolve 5 g of dairy milk (Carnation®) in 50 mL of 1X PBS.
12. 0.05% Tween-20 in 1X PBS (T-PBS)
    - ➢ Dilute 25 μL of TWEEN® 20 (Sigma, cat. No. P5927) in 50 mL of 1X PBS.

- Equipments
    1. Sterile 1.5 mL micro-centrifuge tubes
    2. Sterile 15 mL centrifuge tubes
    3. Sterile 24 well cell culture cluster with flat bottom and lid
    4. 10 μL, 200 μL, 1000 μL auto-pipette and sterile pipette tips
    5. Temperature controllable centrifuge machine
    6. Heidolph Duomax 1030 inclining horizontal rotator for solution mixing
    7. Vortex-genie 2 vortex rotator for solution mixing

Procedures

2.6.1 Find the optimal amount of antibody to be conjugated onto gold nanoparticles

Step 1 – Pipette 5 mL of 100 ppm gold nanoparticles into 15 mL micro-centrifuge tube.

Step 2 – Add 0.4 M K_2CO_3 to gold nanoparticles colloid until the colloid becomes basic (require approx. 50 µL of 0.4 M K_2CO_4). Check the final pH of gold nanoparticles.

Step 3 – Distribute the pH-modified gold nanoparticles into micro-centrifuge tubes.

Step 3 – Prepare varied diluted antibody by *serial dilution* method. 1:2, 1:10, 1:50, 1:100, 1:500, 1:1000 v/v diluted ratio of antibody to final solution are received.

Step 4 – Pipette 2 µL of each diluted antibody solutions and add into pH-modified gold nanoparticles. Put them on horizontal rotator and let them to mix continuously for 15 minutes.

Step 5 – pipette 20 µL of 10% NaCl in DI H_2O and add into each micro-centrifuge tube.

Step 6 – observe color change of antibody-conjugated gold nanoparticles. For the tube which antibody is not enough to cover entire surface gold nanoparticles, its color will change from red to blue.

2.6.2. Functionalization of gold nanoparticles by conjugating specific antibody

(All following steps are completed in low temperature and ice with container is required)

Step 1 – Pipette 1 mL of 100 ppm gold nanoparticles into 1.5 mL micro-centrifuge tube.

Step 2 – Add 10 µL of 0.4 M K_2CO_3 into gold nanoparticles colloid and let the solution to be mixed on vertex rotator.

Step 3 – Pipette 2 µL of optimal diluted antibody into pH-modified gold nanoparticles and leave it to be mixed on horizontal rotator for 15 minutes.

Step 4 – Add 20 µL of 2% PEG in 1X PBS to the tube and continuously mixed for 30 minutes more.

Step 5 – Add 100 µL of 5% BSA in 1X PBS to the tube and mixed for another 15 minutes.

Step 6 – Put the conjugated gold nanoparticles into centrifuge machine and centrifuge for 45 minutes with speed equal to 13000 rpm at 4°C.

Step 7 – When soft gold pellets settle at the bottom and the supernatant solution becomes clear enough, supernatant will be removed until only soft gold pellets left in the micro-centrifuge tube. Then, resuspend antibody-conjugated gold nanoparticles by adds 1 mL of 2% PEG in 1x PBS and mix well using auto-pipette and vortex rotator.

Step 8 – Add 100 µL of 10% BSA in 1X PBS into conjugated gold nanoparticles and let it stand on horizontal rotator to be continuously mixed until used.

Functionalization of gold nanoparticles by conjugating specific antibody

2.6.3 Preparation of antigen on nitrocellulose base to be the testers of conjugated gold nanoparticles.

(All following steps are completed in low temperature and ice with container is required)

Step 1 – Cut small pieces of nitrocellulose membrane (approx. 0.8 x 0.8 cm.) and place them into sterile 24 well cell culture plate.

Step 2 – Dilute antigen as required and well mixed again by vortex rotator.

Step 3 – Pipette 2 µL of diluted antigen and drop onto nitrocellulose base, try to drop uniformly for all antigen.

Step 4 – Let the membrane to dry for a period of time.

Step 5 – Pipette 200 µL of 10% BSA in 1x PBS to block naked surface of the membrane and to stabilize antigen protein for 1 hour at least.

Step 6 – Discard the blocking agent and wash the membrane by 200 µL of 0.05% Tween20-PBS for 15 minutes. Repeat washing for three times at least.

Step 7 – Keep in ice box until used.

2.6.4 Antigen detecting procedure.

Step 1 – Pipette 200 μL of antibody-conjugated gold nanoparticles and fill into each well which antigen on nitrocellulose membrane is presenting.

Step 2 – Put the 24 well on horizontal rotator and continuously move the wells.

Step 3 – When requires time is achieves, remove the conjugated gold nanoparticles out of each wells.

Step 4 – Wash the membrane by adding 500 μL of 0.05% Tween20-PBS to each wells and let it continuously move on the rotator. Repeat washing for three times.

Step 5 – Observe the result on the membrane – red/black dot will appear in case that specific antigen is presenting on the membrane.

However, the experimental results are unsatisfied in some circumstances and the better results are aimed to be pointed out. Thus, we have changed and modified each important condition as much as possible during the project life. For the sake of simplicity, the modified conditions are normally physical or chemical factors rather than biological factors which are harder to find some rare reagents. Physical factors which have been adjusted are centrifuge temperature, length of detecting period, and length of antibody-gold nanoparticles incubation period. While the chemical factors are concentration of antigen, concentration of PEG for resuspending step, and type of chemical substance for blocking reagent to block the naked surface of nitrocellulose membrane and prevent unspecific binding.

The following diagram illustrates the whole process of antibody-conjugated gold nanoparticles includes the variable factors to be adjusted.

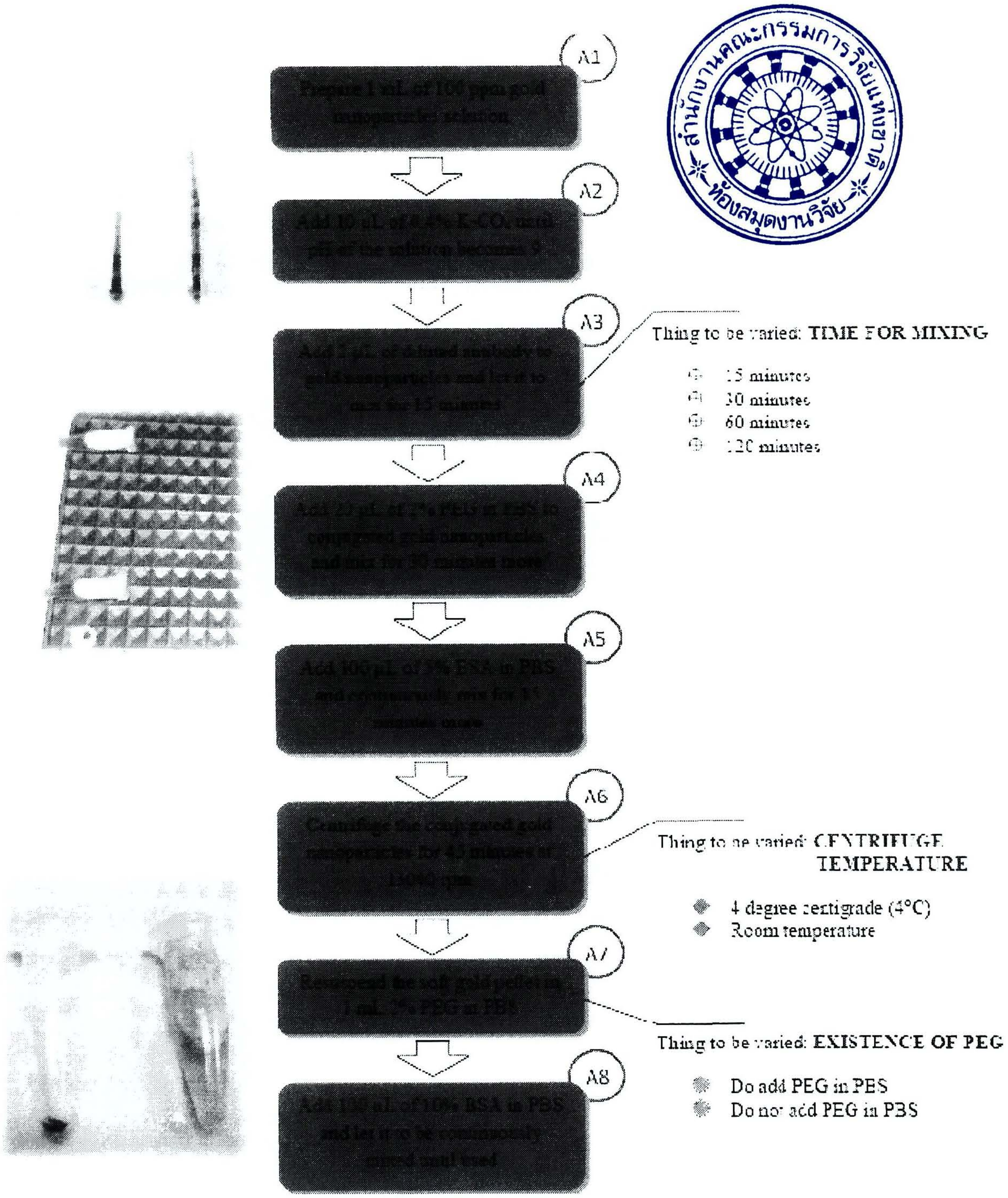


Figure 2.9 : Whole process of antibody-conjugated gold nanoparticles

The following diagram illustrates the whole process of preparation of antigen on nitrocellulose membrane to be used as tester kit in this project. The diagram also includes the variable factors to be adjusted.

Preparation of antigen on nitrocellulose base

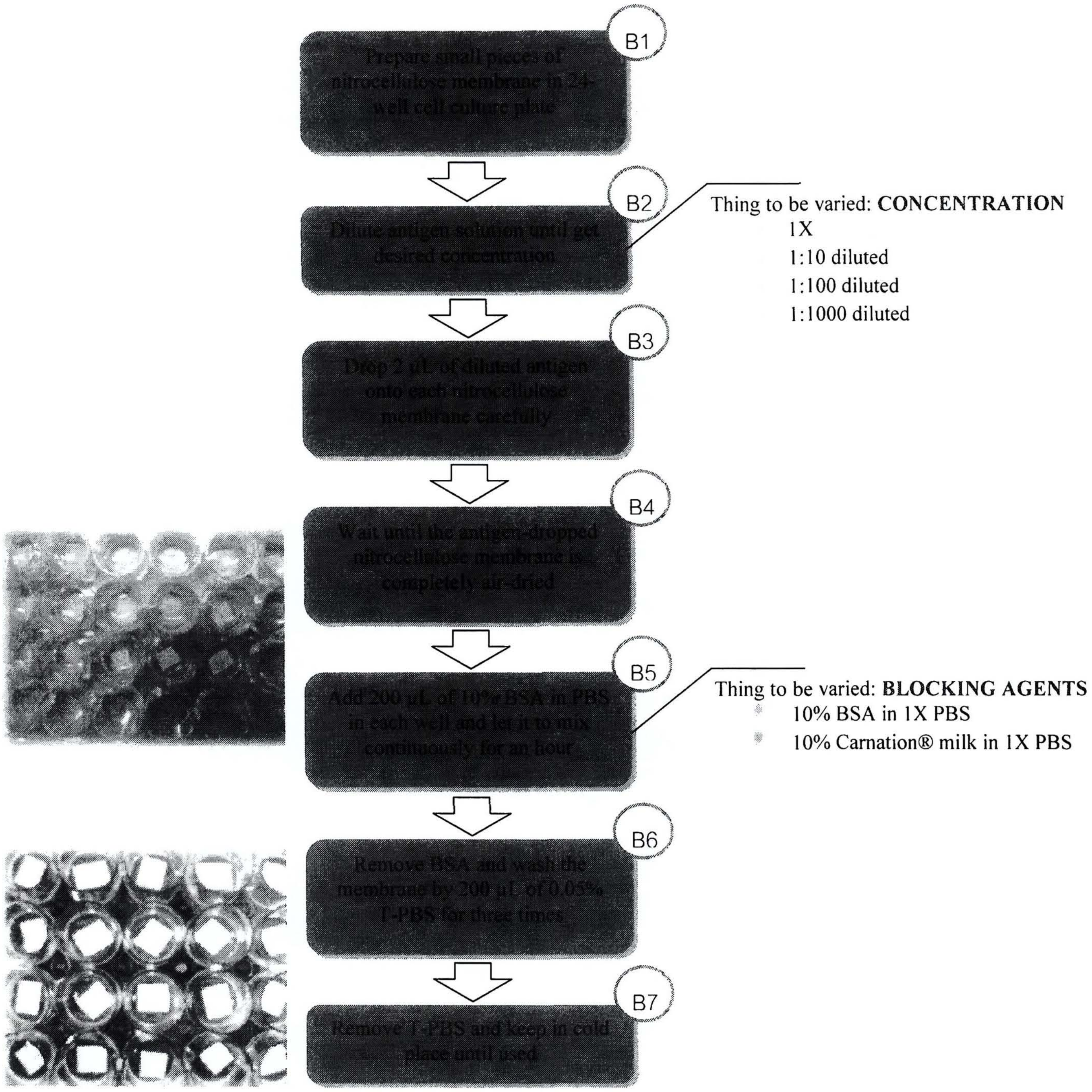
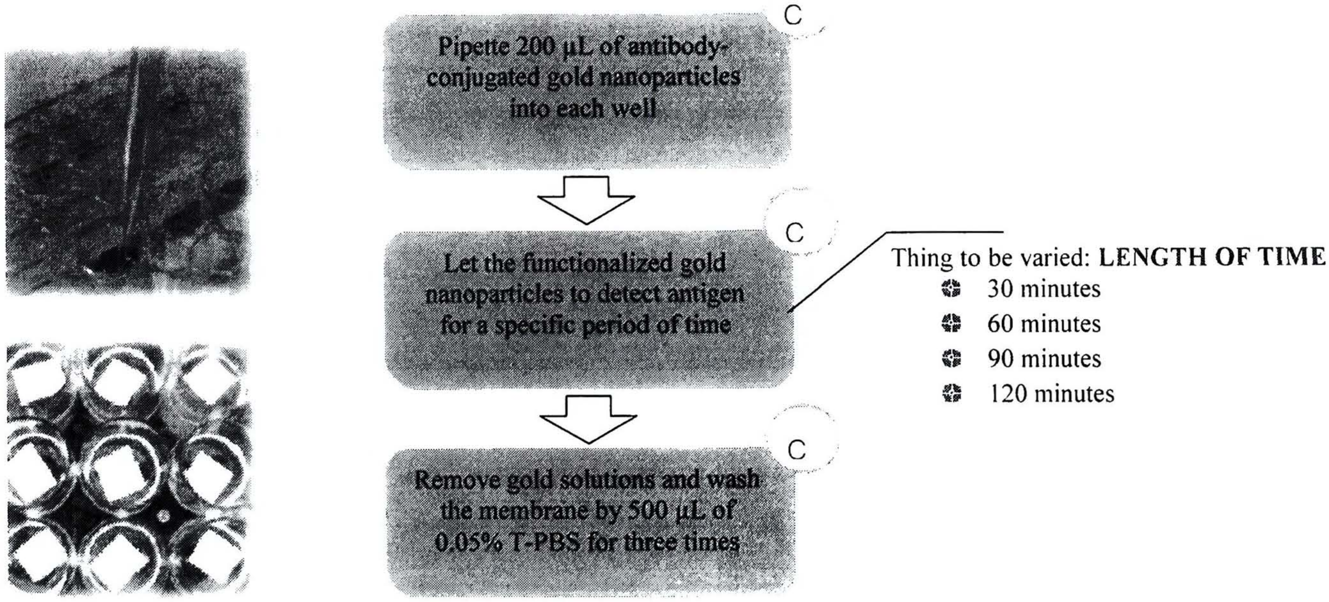


Figure 2.10 : Preparation of antigen on nitrocellulose membrane

The following diagram illustrates the whole process of antigen detecting by using antibody-conjugated gold nanoparticles and washing technique. It also includes the variable factors to be adjusted.

Preparation of antigen on nitrocellulose base



2.6.5 Silver-enhancing to improve signal intensity of conjugated gold nanoparticles

Silver ion with enough light energy source can increase the signal intensity derived from aggregation of gold nanoparticles. Free silver ion in solution can attach onto the dense gold nanoparticles which is locating around specific detectable antigen on nitrocellulose membrane. Attachment of silver ion makes the dot of gold nanoparticles to become darker and easier to be observed by human naked eyes.

Silver solution is synthesized follow the method reported by Chu *et al.*[3] Silver nitrate ($AgNO_3$) is reduced by hydroquinone and precipitate onto gold nanoparticles. The mechanism is explained in physiochemical sense - silver nitrate can be spontaneously reduced by hydroquinone, however, it takes long induction period for reaction to take place. But when gold nanoparticles is presenting, induction period will be decreased because gold nanoparticles can function as catalyst, so, reduced silver (Ag^0) can becomes precipitate cover the area where gold nanoparticles is locating and make the dot from gold nanoparticles to become darker.

- Reagents
  1. 35 mg of silver nitrate (AgNO3)
     - ➢ Taken from pure p. a. grade of silver nitrate (POCH S.A., cat. No. 814322777)
  2. 1 g of hydroquinone
     - ➢ Taken from highly purified grade of hydroquinone (Sigma-Aldrich, cat. No. H9003)
  3. 100 mL of citrate buffer solution (0.243 M $C_6H_8O_7{\cdot}H_2O$ and 0.163 M $Na_3C_6H_5O_7{\cdot}2H_2O$)
     - ➢ Dissolve 4.2 g of citric acid - $C_6H_8O_7{\cdot}H_2O$ (Sigma, cat. No. C1857) and 2.94 g of trisodium citrate - $Na_3C_6H_5O_7{\cdot}2H_2O$ (Merck, cat. No. A661048) in 100 mL of DI H_2O
  4. Deionized water (DI H_2O)

- Equipments
  1. 250 mL Beaker
  2. Hot plate with magnetic stirring function
  3. Magnetic stirrer bar

- Procedure

  Step 1 – Mix 50 mL of citrate buffer with 50 mL of DI H_2O in beaker, continuously stirred.

  Step 2 – Dissolve 1 g of hydroquinone in the solution from step 1 and stir well until all solid form of hydroquinone is totally dissolved. Then, dissolve 35 mg of silver nitrate in the solution from step 2 and observe the color change of the solution.

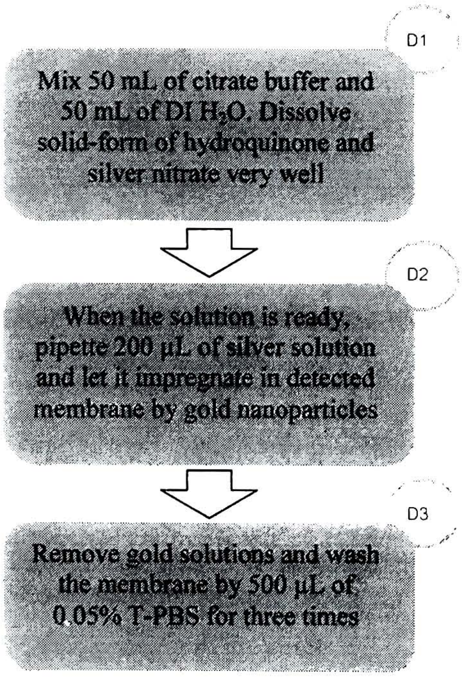
  Step 3 – When the solution turn to be grey, pipette 200 µL of silver enhance solution and add to the nitrocellulose membrane where gold nanoparticles have already attached to antigen and the pink dot of gold nanoparticles is appearing.

  Step 4 – Let the silver to impregnate in dark for a period of time.

Step 5 – Remove the silver solution and wash the membrane by 0.05% T-PBS solution for 3 times.

Step 6 – Observe the result on the membrane.

Figure 2.11: Usage of silver solution to enhance signal from gold nanoparticles



2.7 Detection of Leptospira in urine using anti-Leptospira-coated AuNPs

Recently, nanoparticle-based assay has been introduced as a tool for both disease therapy and laboratory diagnosis. The detection of Salmonella spp. Using antibody-coated gold nanoparticles and dielectrophoretic impedance measurement has been reported [17]. Here we demonstrate that Leptospira antibody-coated gold nanoparticles can be used for urine Leptospira detection as a model for using antibody-coated AuNPs for FUO diagnosis

2.7.1 Bacteria and antibody

The reference Leptospira interrogans serovar Bratislava was kindly provided by WHO/FAO/OIE Collaborating Centre for Reference & Research on Leptospirosis, Brisbane, Australia. Rabbit polyclonal antibody specific to Leptospira interrogans serovar Bratislava was purchased from Royal Tropical Institute, Amsterdam, Netherlands. Escherichia coli, Klebsiella pneumoniae, and Enterococcus faecalis were obtained from the Department of Microbiology, King Chulalongkorn Memorial Hospital.

2.7.2 Preparation of gold nanoparticles

The 9-nm gold particle suspension was prepared according to Turkevich citrate reduction method . Briefly, 1ml of 12.7mM HAuCl4 (Sigma, MO) was mixed with 49 ml deionized distilled water. The solution was then heated at 100 8C on the magnetic stirrer until boiling. 0.94 ml of 38.8mM sodium citrate was added until the color of gold suspension turned red. The solution was boiled for 5 min further, left to cool down at room temperature and kept for future use at 4 8C.

2.7.3 Preparation of Leptospira antibody-coated gold nanoparticles

Antibody-coated gold nanoparticles were prepared according to previous reports [19,20]. Briefly, the pH of gold nanoparticle solution was adjusted by adding 0.2 mol/LNa2CO3. Five microliters of antibody specific to Leptospira interrogans serovar Bratislava was incubated with 45 ml gold nanoparticle suspension at room temperature for 30 min. The mixture was then treated with 16.7ml of 10% bovine serum albumin (Sigma) at room temperature for 10 min and washed with washing solution (1% bovine serum albumin, 0.1% NaN3 in 0.02M sodium borate) by centrifugation at 25,000 _ g for 30 min. The pellet was resuspended in 50ml of washing solution. Antibodycoated gold particles can be kept at 4 8C for further use.

2.7.4. Detection of urine Leptospira by antibody-coated gold nanoparticles

Two hundred microliters of urine containing Leptospira was centrifuged at 20,000 g for 15 min at 4 8C. The sediment was resuspended in 20ml deionized distilled water and then mixed with 50ml antibody-coated nanoparticles. Agglutination of red gold particles indicates positive result.