Progress report on the rapid test kit development for early detection of systemic lupus erythematosus in Indonesia

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Abstract. Gold standard diagnosis for Systemic Lupus Erythematosus (SLE) is ANA and anti-dsDNA antibody test using ELISA. We are developing rapid test kit for early detection of SLE which is affordable and ease by blotting antigen into membrane since 2015, yet the previous trial did not produce good result. Our recent trials aim to optimize antigen isolation and its attachment to membrane. This was trial-and-error experiment using healthy unused human transfusion blood and manual (non-kit) isolation methods. We compare 2 methods each for leukocyte isolation, nuclear antigen isolation, dsDNA extraction and antigen attachment to membrane to produce colour differences in the final kit. Leukocyte buffy coat is thicker using single sample human transfusion blood compared to pool multiple sample, and the quantity was comparable in both isolation methods. More nuclear protein yield was achieved through the use of Nuclear (NE) and Cytoplasmic Extract (CE) buffer. From 15 cc of whole blood, can be produced 3-5 µL/µL protein. Modified salting out method was using high concentration salt and extra 0.05 mg/mL Proteinase-K result in more yield, 700-800 µg/µL of dsDNA. Attaching dsDNA was feasible using PVDF membrane, by applying 200 µg antigen in each blot and BSA 3% blocking.

1. Introduction
Systemic lupus erythematosus (SLE) is a disorder in which immune system mistakenly attacks its own healthy cells and tissue, known more frequently in productive aged-women. Eighty percent patients are female between adolescence and fourth decade of life, and characterized by widespread of inflammation [1,2]. Pathogenesis of this disease are not clearly understood. It is known that immune system deregulation in lupus patients causes secretion of pathogenic auto-antibodies or autoimmune and manifested in tissue damages [3]. Diagnosing SLE is quite difficult because of its atypical clinical symptoms and need a laboratory test to detect auto-antibodies. ANA and anti-dsDNA are highly sensitive and specific marker for SLE [4]. Both antibodies can be found in 40-90% of patients with active disease [5]. Available commercial kits are using ELISA method. This type of test is not feasible to be done in rural hospital around Indonesia, due to the need of trained personnel and additional equipment [6,7].
Indonesia is known with high mortality and complications in patients due to delayed diagnosis of SLE. Therefore, early diagnosis for antibody testing is necessary to solve the disease burden in this country [8]. The purpose of this research is to develop a rapid diagnostic kit in the strip (membrane) format to detect antibodies toward nuclear antigen and double strand DNA in serum patients with suspected SLE. This kit has to be easy to use, affordable and sensitive. This project has been started since 2015, comparing antigen sample from HeLa cells, hair follicle and human blood using the commercial isolation kit. Unfortunately, the method to determine positive and negative values have not been discovered yet, the research cost have been spent enormous. This time, we are using whole blood sample from unused transfusion blood stock provided by Red Cross Indonesia. All isolation steps were not performed using the kit, but manual techniques using self-made buffers to optimize antigen extraction and attachment.

2. Material and Methods

This was a trial-and-error laboratory experimental research, conducted from May to November 2017 in two locations: The Biomedical Central Laboratory in Medical Faculty of Brawijaya University, and the Central Laboratory of Brawijaya University. This study protocol is continuation of previous research which was approved by Health Research Ethics Committee, Medical Faculty of Brawijaya University. We perform isolation and purification of nuclear protein antigen and DNA using donated blood from the Indonesian Red Cross Society. Acquired antigens were tested against antibody from serums of ANA-positive, ds-DNA-positive, ANA-negative, ds-DNA-negative SLE patients. The flowchart below describes overall comparison methods used in this experiment (Figure 1).

![Flowchart of the experiment](image)

2.1. Isolation of Leukocyte

In isolation of leukocyte experiments using ficoll solution and Ficoll. Prepared in whole blood in EDTA, A layer of whole blood put on top of Ficoll 1:1. Centrifuge 1600rpm for 30 minutes. Harvest the Buffy coat, washed with PBS by 1000 g centrifuge Harvest pellet.

A trial using Ery-Lysis Buffer

The ingredients needed include \( \text{NH}_4\text{Cl} \ 8.26 \text{ g} ; \ \text{NaHCO}_3 \ 1.19 \text{ g} ; \ 0.5 \text{ M EDTA} \ 200 \mu \text{ l} ; \ 100 \text{ cc sterile water.} \) The materials are diluted first and made 10x Ery-Lysis buffer. Adjust pH to 7.3, then filter for sterilization. Prepare whole blood in EDTA, mixed 5cc of blood with 15cc Ery Lysis buffer (1:3). After that be shaken well for 5 minutes and incubated in 4°C for 10 minutes. The solution centrifuged in 5000rpm for 10 minutes at room temperature, repeated again the procedure until the pellet is clear from the erythrocyte.
2.2. Isolation of Nuclear Protein

A trial using Cytoplasmic Extract (CE) and Nuclear Extract (NE) Buffers

The materials used are CE Buffer for 50 cc: 0.5 cc HEPES 1 M, 1.25 cc KCl 2 M, 10 μl EDTA 0.5 M, NP-40, and Protease inhibitor. NE Buffer for 50 cc: 4 cc NaCl 5 M, 1 cc HEPES 1 M, 100 μl EDTA 0.5 M, 12.5 cc glycerol and protease inhibitor. The materials are added CE buffer and Tripton 5 times estimated pellet. Incubated on ice and homogenized for 5 minutes. Centrifuged in 3000 rpm, for 5 minutes at 4 °C. Harvest supernatant, this is the cytoplasm. CE added without Tripton 100 ul in to nuclear pellet. Centrifuged 3000 RPM, 5 minutes at 4 °C. NE buffer 1:1 added. Resuspend, incubated 10 minutes on ice and homogenized. The resuspension centrifuged on 14,000 RPM for 5 minutes at 4 °C. Harvest supernatant. Storage in temperature -40 °C.

A trial using Salt Buffers

The material used low-salt buffer: 10 mM HEPES-KOH pH 7.90, 1.5 mM MgCl$_2$, 0.1 mM EDTA, 10 mM KCl; High-salt buffer: 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 20 mM HEPES-KOH, 25% glycerol, protease inhibitor; Sucrose buffer: 1.5 M sucrose, 10 mM HEPES-KOH, 1.5 mM MgCl$_2$, 10 mM KCl, 0.1 mM EDTA, Protease inhibitor; Hypo-osmotic lysis buffer: 10 mM HEPES-KOH, 1.5 M MgCl$_2$, 10 mM KCl, 0.3 M sucrose, 2% Tween, protease inhibitor. All buffers, sample and tube should be chilled before the procedure, all centrifuges in 12,000 RPM 4 °C. Prepared leukocyte pellet and washed with PBS for 30 seconds. Estimated volume, and added hypo-osmotic lysis buffer 5x. Homogenized first and added 1 cc of Sucrose buffer 1.5 M, centrifuged for 10 minutes. The Harvest pellet, added 1 cc of Low salt wash buffer, centrifuged for 30 seconds. Harvest pellet, added 50 μl of High Salt Extraction buffer, incubated in ice for 20 minutes, homogenize and centrifuged for 20 minutes, the harvest supernatant. Storage in temperature in -40 °C.

2.3. Isolation of double stranded DNA

A trial using Original Salting Out method

The materials used K-buffer for 250 μl: 0.05 M KCl, TritonX100, 0.01 M Tris-Cl, 0.05 mg/ml Protease K, 2.5 M NaCl, 0.01 M Tris-Cl, ethanol. K buffer added in to leukocyte pellet, incubated overnight at room temperature or 1 hour in 55 °C. NaCl 250 μl of 2.5 M added and vortex vigorously for 15 seconds. Centrifuged 10,000 RPM for 2 minutes. Absolute ethanol 1 cc added, harvest DNA. Ethanol 70% added, harvest DNA and placed in 100-200 μL of sterile water.

A trial using Modified Salting Out method

The materials used SE Buffer 10x: 43.8 g NaCl, 89.3 g EDTA, 11.0 g NaOH, 800 cc water, adjust pH to 8.0. PBS 0.05 mg/mL protease K, 2.5 M NaCl, 0.01 M Tris-Cl, ethanol. K buffer added in to leukocyte pellet, incubated overnight at room temperature or 1 hour in 55 °C. NaCl 250 μl of 2.5 M added and vortex vigorously for 15 seconds. Centrifuged 10,000 RPM for 2 minutes. Absolute ethanol 1 cc added, harvest DNA. Ethanol 70% added, harvest DNA and placed in 100-200 μL of sterile water.

2.4. Attaching Antigen to Membrane

A trial using Nitrocellulose Membrane

The material used dot blot apparatus; nitrocellulose membrane; TBS: Trisbase 1.212 g, NaCl 2.336 g, Aquadest 200 cc, adjust pH 7.4. Tween20. Blocking low fat milk 0.5 g. The procedure activated membrane in TBS, in every blotting put 50 μL each well, washing up to 3 times used TBS Tween. Diluted antigen in TBS, attach to the membrane by vacuum. Put blocking solution, incubated at 37 °C.
minutes 37°C, washed and added primary antibody, incubated overnight 4°C, and washed. Then added secondary antibody, incubated one hour 37°C and wash. Finally, added substrate for 45 minutes in a dark room. Washed and interpret result.

A trial using PVDF Membrane
The materials used PVDF membrane preparation: dip in methanol 15 seconds until transparent, dip in aquadest for 2 minutes, TBS 5 minutes, layer with filter paper dipped in aquadest. all blotting materials are diluted in TBS Tween. Blot antigen, let air dry, baked 80°C for 1 hour, expose with UV light 30μJ. Added primary antibody 1:40, incubated 15 minutes 37°C and washed. A secondary antibody added and incubated 15 minutes 37°C and wash. Added streptavidin and waited for 15 mins 37°Cand then washed. After that added substrate for 5 minutes and interpreted results.

3. Results and Discussion
SLE is an autoimmune disease with varies clinical symptoms therefore very broad and difficult to recognize. One characteristic of SLE is the production of auto-antibodies directed against nuclear antigens such as dsDNA, nuclear, nucleoli, histone, chromatins. Therefore, ANA and anti-dsDNA titer is important marker used in the diagnosis and evaluation of SLE. It is known that this antibody can be found in 40-90% of SLE patients with active disease. This auto-antibody manifest in immune complex mediated organ damage. Delay in diagnosis and treatment can lead to increased morbidity and health care cost. The concept of antigen and antibody interaction is used for the development of this diagnostic kit to provide an early detection of disease.

There are several diagnostic kits for autoimmune disease that is quick and easy to perform such as rheumatoid arthritis (RA). This kit contains antigen, which will bind to the autoantibodies produced by RA patients with a good specificity and sensitivity. Meanwhile, rapid test kit for SLE is not widely developed due to numerous types of antibodies detected. Also, antibody profiling has never been done in Indonesia. One research mention that the most frequently reported autoantibodies in Indonesia patients with SLE are ANA and anti-dsDNA.

In this study, isolation and purification of nuclear and DNA antigens using human blood as a sample. We compare 2 samples, first is pooled whole blood from multiple healthy donor, and the second is whole blood from single healthy donor taken from the unused blood transfusion pack. Whole blood should be stored at 4°C and be processed in less than 5 days to achieve a good amount of leukocyte.

We found through Ficoll solution method, the Buffy coat became very thin when multiple samples are pooled. This can be caused by cell lysis due to plasma antibody reacts toward surface protein antigen on cell from another mixed sample. Due to high production need, we are using a single sample with large volume blood which is transfusion pack. The unused whole transfused blood is supplied by the Red Cross organization. In most cases, urgent and high-risk surgery requires blood stock to be used during operation if needed. If not, the thawed blood will be wasted. Rather than be wasted, we’re using it in the lab.

We also compare ficoll and Ery-lysis buffer to isolate leukocyte. We found no significant difference in leukocyte pellet volume, around 50μl from 15cc of blood. Ficoll is on one side faster but more expensive. Ery-lysis buffer is easy to create and cheap, but takes longer time to isolate due to repeated procedures. Whole blood composed of cellular and plasma. Plasma contains water and soluble protein, including clotting factors. Cellular consists mostly erythrocyte, platelets and leukocytes (such as lymphocyte, monocyte, and granulocyte). Each has a different molecular weight, structure and quantity. Since we are aiming for nuclear protein and DNA, the cell carries both materials are ones with a nucleus, which is leukocyte.

There are two ways to isolate leukocytes, one by separate cell using its weight (through Ficoll-hypaque purification) or breakdown other cells and seclude leukocyte (through ery-lysis buffer). Many other methods are available for choices, in this case we compare two most feasible and affordable methods. Ery lysis buffer, like its name, act by destruct erythrocyte membrane through chemical and
mechanical techniques. Erythrocyte membrane becomes fragile with high salt concentration, using ammonium chloride, sodium bicarbonate also EDTA. Lysis process continued by mechanical disruption using repeated manual shaking, and centrifugation in high temperature. Using this procedure, platelets will be concentrated in the supernatant. From Yazigi et al research (2015), centrifugation 410g for 10 minutes, causing platelets to be aggregated in serum up to eleven times higher yield.

Ficoll-Hypaque purification is on the other hand separating each cell population without destructing its structure. Centrifugation on its procedure is done at one time, slower speed (1600-2000rpm), but enough to move around cells and create layers based on its specific gravity. This is very slow compared to other method using up to 5000rpm centrifugation, two to three times repetition. In previous method, the harvested leukocyte is in the pellet, but using Ficoll we are harvesting buffy coat, a layer formed between Ficoll and plasma, which consist of pooled lymphocytes. Due to its molecular weight, granulocytes are not included in the buffy coat, but settle with erythrocyte in base layer.

The next step is isolating antigen. There are 2 antigens will be attached to the membrane. One is a nuclear protein to replicate ANA, and the other is dsDNA. To isolate nuclear protein, we compare 2 types of buffer. Both buffers are created from scratch. We also compare using 2 types of Eppendorf, which is filtered and non-filtered. The result is protein yield which is quantified by nanodrop. Table 1 showed the result of protein yield. We found protein yield can be produced in higher yield and more consistent using cytoplasmic (CE) and nuclear extract (NE) buffer and non-filtered Eppendorf.

Protein in the cell nucleus can only be extracted when the cell membrane to the nuclear membrane breaks. This can be achieved using mechanical force (sonication, pipetting, homogenization, and centrifugation) and non-mechanical such as detergent, salt, or combinations to create a pressure difference inside and outside the cell, which then release the protein. In every procedure, we must be cautious that protein can be easily denaturation in high temperature. Therefore, it is necessary to provide ice cube to chill samples in every step. Since both procedures require manual recipe, it is important to have the buffer fresh, or recheck its pH and clarity before using stock buffers. Several times we stumble upon low protein yield caused by contaminated buffer. There was no exact reference on how much proteinase inhibitor tablet should be used for buffer. Adding it freshly before isolation is important to avoid enzyme (proteins) which will break down our sample during cell lysis. Both procedures in this experiment are using detergents (Tripton, NP40, and Tween) and salt to break leukocyte. We found that CE and NE buffer is easier, faster and produce more yield than the other method. This difference is basically caused by different composition and exposure times to detergent. Although there are highly advanced methods to extract and purify proteins, it is hard to produce more than milligram quantities of nuclear protein.

Table 1. Isolation of Nuclear Protein Antigen.

| Yield (ng/μl) | Buffer Type | Eppendorf Type |
|--------------|-------------|----------------|
| 4670         | A           | Non-filter     |
| 2000         | A           | Non-filter     |
| 930          | A           | Non-filter     |
| 280          | B           | Non-filter     |
| 50           | B           | Non-filter     |
| 30           | B           | Non-filter     |
| 620          | A           | Non-filter     |
| 170          | B           | Filter         |
| 420          | A           | Non-filter     |
| 928          | A           | Non-filter     |
| 281          | A           | Filter         |

A: Trial using Cytoplasmic (CE) and Nuclear Extract (NE) Buffers
B: Trial using Salt Buffers
In isolating and extracting double strand DNA, we compare two methods which are original salting out and modified salting out. The difference is in cell lysis solution and detailed procedure. The result of DNA yield is presented on Table 2. It can be concluded that DNA can be produced in higher purity and yield through modified salting out method.

Basic step on both salting out method (original and modified) is to break the leukocyte (or lymphocyte) membrane, precipitate its protein then precipitate also purifies DNA. Membrane lysis is again achieved with salt and detergent. During this process it’s important to avoid our antigen destroyed by nucleases (DNAse and RNAse), therefore adding protease-K is crucial step in buffer making.\(^{19}\) High concentration salt is used to precipitate protein (from cell lysate). It varies from 2.5 to 5.0 M of natrium chloride. We found that 2.5M of salt, 1:2, is enough to create protein precipitate, marked by white-brownish pellet forming after centrifugation. This salt solution does not only precipitate protein but also bounds DNA. In the next step, purifying DNA from sodium chloride can be achieved using ethanol. Using pre-chilled ethanol in gradient matter (absolute ethanol then 70% ethanol), will produced visible DNA strands floating on the upper layer of this solution. Based on Smarason and Smith method, combining phenol, alcohol and chloroform can also be done to extract DNA with ratio 25:1:24.\(^{20}\) To avoid ethanol contamination in our sample, after harvesting DNA, let the ethanol evaporates in room temperature, and then dissolve our sample in TE buffer or sterile distilled water.\(^{21}\)

| Purity | Yield (ng/μl) | Salting Out Method |
|--------|--------------|--------------------|
| 1.6    | 41.6         | Original           |
| 1.58   | 59.9         | Original           |
| 1.62   | 50.5         | Original           |
| 1.85   | 724.6        | Modified           |
| 1.84   | 897.0        | Modified           |
| 1.83   | 447.0        | Modified           |
| 1.74   | 479.3        | Modified           |
| 1.85   | 8267         | Modified           |
| 1.92   | 354.2        | Modified           |
| 1.93   | 363.2        | Modified           |
| 1.90   | 382.8        | Modified           |
| 2.11   | 155.8        | Modified           |
| 2.19   | 128.7        | Modified           |
| 1.95   | 199.7        | Modified           |
| 2.08   | 141.8        | Modified           |
| 2.18   | 184.8        | Modified           |
| 2.01   | 235.8        | Modified           |

Final step is attaching antigen, nuclear protein and dsDNA into membrane. There are 3 types of membrane commonly used for direct blot or transferred following gel separation, those are nitrocellulose, PVDF and nylon.\(^{22}\) we compared 2 types of membrane which is nitrocellulose and PVDF. During the checker board test, both nuclear and ds-DNA might show colour change (Figure 2). Checkerboard test is using antigen (nuclear protein, ds-DNA), binds with positive antibody from human serum to search the best dilution ratio for each sample. Unfortunately, this did not happen when we test with positive and negative antibody. Both nuclear and ds-DNA dot blotting on Nitrocellulose membrane did not show the difference colour between controls, positive, and negative (Figure 3 and Figure 4). Nitrocellulose is hydrophobic membrane, and with lower binding capacity. It is good for nucleic acid analysis, but known can only attach single strand DNA and not double strand DNA.\(^{23}\) In this case, there are many factors can affect this failure. First whether the antigen (nuclear protein and DNA) is firmly attach to the membrane. We did not do SDS Page to separate protein because there’s no specific reference on which protein does the autoantibody binds to in SLE. Since protein molecular weights were not determined, it might be possible that the quantity of protein blotted is reduced due to leakage from the membrane.
Figure 2. Checkerboard of nuclear antigens in Nitrocellulose membrane.

All antigens showed colour change when reacted to positive serum. Column represents antibody dilution and row represents antigen dilution. 1: 1/50; 2: 1/100; 3: 1/200; 4: 1/400; 5: 1/800; 6: 1/1600; 7: 1/3200; 8: 1/6400; 9: 1/12800; 10: 1/25600; 11: 1/51200; 12: control; A: 0.15 μg/μl; B: 0.07 μg/μl; C: 0.04 μg/μl; D: 0.02 μg/μl; E: 9 ng/μl; F: 4 ng/μl; G: 2 ng/μl; H: 1 ng/μl. Black framed dot showing adequate color change with lowest antigen and antibody dilution (1/12800 and 2 ng/μl).

Miribel and Arnaud (1988) did also face the same problem in their western blot on nitrocellulose membrane, and it cannot be solved by modifying its buffer alone, but need a different membrane for better binding which is nylon. Dunn (1986) also found that addition of Tris-HCl and pH adjustment for antigen blotting correlates with better binding of small molecular weight protein.

Figure 3. Dot Blot assay of nuclear antigens in Nitrocellulose membrane.

The dot blotting did not show the difference colour between control, positive, and negative. Column represents antigen dilution and row represents antibody dilution. 1-6: 1 ng/μl; A, E: 1/100; B, F: 1/3200; C, G: 1/6400; D, H: 1/12800; Red framed dots are tested with positive ANA test serum, and Green framed dots are tested with negative ANA test serum.
The dot blotting did not show the difference colour between control, positive, and negative. Column represents antigen dilution and row represents antibody dilution. A,D: 4ng/μl; B,E: 2ng/μl; C,F: 1ng/μl; 2, 6, 10top3: 1/50; 3,7,11: 1/6400; 4,8, 10bottom3: 1/12800; Red framed dots are tested with positive ds-DNA test serum, green framed dots are tested with negative ds-DNA test serum, and yellow framed dots are control.
Then we tried to attach both antigens on PVDF membrane. Again, after positive and negative test, we found no colour difference in nuclear protein antigen (Figure 5). But there was colour difference between positive and negative anti-dsDNA test, unfortunately the colour fades after the membrane dry (Figure 6). One main structural difference between PVDF and nitrocellulose membrane is its binding capacity. PVDF is known to have higher binding capacity, therefore is recommended for western blotting. This ability comes in both advantages and challenges. First, attaching antigen in this membrane will give better exposure for antigen-antibody binding, especially if the sample used has low protein expression. Unfortunately, if the quantity of antigen blotted is too limited, there will be blank parts exposed and cause higher chance of background (unwanted substrate binding). In our trials, we tried several blocking solutions such as bovine serum albumin (in 2-5% concentration) and skimmed milk. Colour changes in dsDNA membrane (although it fades) were achieved using bovine serum albumin 5%. Other factors needed to be confirmed is whether the protein displaced after blotting due to detergent use during washing. In the method we were using tween20 for PVDF washing buffer, a trial using no detergent, or modify detergent therefore is essential. Baldo (1986) did compare several detergents for nitrocellulose membrane, and found tween does not ruin antigen-antibody binding, but we found no similar data for PVDF membrane. In this experiment, after antigen blotting, the membrane was let dry in room air before baking the membrane. We found this method is shows better result than vacuum. Polvino (1983) stated the same method for protein blotting in nitrocellulose membrane, but no similar data for PVDF membrane is found.

The dot blotting did not show the difference color between positive and negative. Column represents antibody dilution and row represents antigen dilution. 1, 6: 1/25; 2, 7: 1/50; 3, 8: 1/100; 4, 9: 1/200; 5, 10: 1/400; A: 10ng/μl; B: 5ng/μl; C: 2.5ng/μl; D: 1.25ng/μl; E: 0.625ng/μl; F: 0.3125 ng/μl; G: 0.1562 ng/μl; H: 0.0781 ng/μl. Red framed dots are tested with positive ANA test serum, and Green framed dots are tested with negative ANA test serum.

4. Conclusion
The cost of producing this kit has been suppressed by using manual technique. There are multiple factors that needed to be optimized in further research, such as sample storage and isolation method to produce more protein yield, also modify DNA-antibody-substrate binding so color change can stay in longer period of time. It is hoped that early lupus enrolment in primary health care centres can be improved with the discovery of this lupus diagnostic kit. This in turn will enable the management of lupus as early as possible so as to reduce mortality and improve patient’s survival rate.

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