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Development of nucleic acid based lateral flow assays for SARS-CoV-2 detection

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SARS-CoV-2 is still threat for humanity and its detection is crucial. Although real time reverse transcriptase polymerase chain reaction is the most reliable method for detection of N protein genes, alternative methods for molecular detection are still needed. Thus, lateral flow assay models for 2019-nCoV_N3 were developed for molecular detection. Briefly, gold nanoparticles were used as label and three sandwich models (1A, 1B, and 1.2) were designed. Prob concentrations on gold nanoparticles, types of sandwich model and membrane, limit of detection of target gene and buffer efficiency were studied. Model 1B has shown the best results with M170 membrane. Lower limit of detection was achieved by model 1.2 as 5 pM. All parameters have significant role for molecular detection of SARS-CoV-2 by lateral flow assays, and these results will be useful for nucleic acid based lateral flow assays for viral detection or multiple detection of mutated forms in various detection systems.

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Wuhan viral pneumonia seen in the late 2019 was called as SARS-CoV-2 and COVID-19 caused by SARS-CoV-2 was named as disease by World Health Organization (WHO). SARS-CoV-2 is an RNA virus belonging to the β and it has at least four structural proteins including spike (S) protein, envelope (E) protein, membrane (M) protein and nucleocapsid (N) protein. S protein is widely used for diagnosis because of antigenicity and commonly chosen as target for neutralizing antibodies. However, N protein becomes attractive for molecular diagnosis of COVID-19 as highly protected protein sequences and high immunogenicity (1). Additionally, N protein is abundantly expressed protein during the infection and causes to protective immune response for SARS CoV and COVID-19 (2). The sequence similarity of protein coding region of COVID-19 was found as 89.74%, 48.59% and 35.62% with SARS CoV, MERS-CoV and HCoV-OC43, respectively (2) and 96% with bat coronavirus in the whole genom level (1). The comprehensive domain structure of N proteins among the four coronaviruses (SARS-CoV-2, SARS-CoV, MERS-CoV, and HCoV-OC43) and the complete genome of SARS-CoV-2 were reported in the literature (3). To say that the characteristics of the surface electrostatic potential of N terminal domain of SARS-CoV-2 N protein is different even if it is similar to the other coronaviruses.

N region of SARS-CoV-2 was determined as target sequence for SARS-CoV-2 specific gene. WHO proposed a few primer sets for N gene and reported that 2019-nCoV_ N3 (USA) and NIID_2019-nCOV_N (Japan) primers are the most sensitive for real time reverse transcription polymerase chain reaction (rRT-PCR) (4). Therefore, among the specific regions N gene regions are widely accepted for diagnosis as the high similarity between SARS-CoV-2 and SARS-CoV causes to mistake in molecular diagnosis. SARS-CoV-2 RNA may be obtained from bronchoalveolar lavage, nasal/pharyngeal swab (53.6%–73.3%) (5), saliva/sputum (74.4%–88.9%) (6), feces/urine, blood samples and anal/oral swabs (7–9). Additionally, it is known that virus may be alive at suitable environmental conditions after leaving from human body and join to waste water. Thus, SARS-CoV-2 and newly developed coronaviruses will always threaten the public health since the development of antiviral drugs or therapeutics takes long time. In this reason, early molecular viral detection is crucial to get under control the epidemic/pandemia.

Serology is a standart method for viral detection and based on the testing of antibody response coming from the immune system and antigen presence. However, it cannot be used for early detection since it is based on the measurement of antibody after infection, and could not be efficient for patients who are in risk groups. For instance, antibodies against to COVID-19 are developed in early stage (4–10 days for IgM) and late stage (11–24 days for IgM–IgG). Besides, cross antibody reactions may give a false positive result and producing of polyclonal antibodies may change from batch to batch. Nanoparticle based viral diagnosis (10) is another way and it was used for the detection of SARS specific sequence (11). However, there is lack of nano-based diagnosis systems for SARS-CoV-2 sequences even if antigen based detections are reported (12–17). rRT-PCR is the most reliable method for molecular detection of SARS-CoV-2 in the world and the first quantitative rRT-PCR was designed after the definition of virus by WHO in January 2020. Although these assays are reliable, complex and expensive test protocols, need of educated personnel and diagnosis laboratories, taking time for sending the samples into reference labs are disadvantages. Similarly, conventional PCR needs agarose gel loading and high copy number of target genes. For these reasons, rapid and
naked-eye molecular detection of SARS-CoV-2 is always needed. In this regard, lateral flow assays (LFAs) or point of care tests could be an alternative to the molecular detection of SARS-CoV-2 as a rapid, cheap and simple way without advanced devices in a short time. LFAs are portable, ready to use immunochromatographic diagnostic assays developed by antibodies, enzymes or nucleic acids (18) for various fields. They can also be helpful for epidemi/pandemia by sensitive detection of nucleic acids (19) and could make 8 times sensitive and rapid detection compared to the electrophoresis (20). While a number of LFAs were developed from COVID-19, they are mostly based on the antibody (IgG/IgM) section of patients (21) and there is lack of nucleic acid detection of SARS-CoV-2 by LFAs. LFAs can be used with amplification systems producing any RNA (22). Although LFA for molecular detection of SARS-CoV-2 is reported in the literature, it is based on CRISPR Cas12a dependent nucleic acid detection (23) and needs the complex experimental steps. Similarly, Broughton et al. (24) developed the LFAs for SARS-CoV-2 using the RNA extracts related with respiratory swab. It is based on the CRISPR—Cas12 for the detection of E and N gene (24). However, extra labelling with fluorescein amides and sensitive steps including enzymatic restriction are needed, and assay was only developed for one region of N gene announced by US Centers for Disease Control and Prevention which are accepted regions for rRT-PCR. LFAs are encouraged to be developed for nucleic acid detection of SARS-CoV-2 based on PCR. Therefore, nucleic acid-based LFAs that can adopt new sequences generated by viral mutations are always important.

In this research, molecular detection of RNA region (2019-nCoV_N3) specific to SARS-CoV-2 by gold nanoparticles (AuNPs) based LFAs in 5–7 min was aimed. Test principle is based on the hybridization of oligonucleotides without complex enzymatic reactions and naked-eye analysis.

RESULTS AND DISCUSSION

Synthesis of gold nanoparticles and conjugation with oligonucleotide probes
Synthesized AuNPs were analyzed by STEM, UV–Vis spectroscopy and Dynamic Laser Particle Size Analyzer. According to the analysis, homogeneously distributed spherial colloid AuNPs were measured as about 13 nm and \( \lambda_{\text{max}} \) was 521 nm as expected (Fig. S1). Additionally, the measurement of STEM analysis showed that value: 1 \( \text{nm} \), objective count: 85, summation: 1071.01, minimum value: 9.44, maximum value: 20.26, mean value: 12.60 and standard deviation: 2.21. The concentration of synthesized AuNPs was also calculated as 0.4 nM according to the extinction coefficient of 13 nm at 521 nm wavelength (27).

After the conjugation of AuNPs with thiol modified probes, their max absorption peaks were shifted from 521 to 526 nm as expected (Fig. S2). This is because in the three sandwich models, the coating of AuNPs by the probes (4 \( \mu \text{M} \) and 8 \( \mu \text{M} \)) changed the surface charge of the AuNPs and shifted the maximum absorption peak. While the concentration of probes on AuNPs was enough for 4 and 8 \( \mu \text{M} \) for three models, 2 \( \mu \text{M} \) probe was not enough for sustaining the stability of AuNPs since it caused the aggregation of AuNPs [data not shown]. Therefore, 4 and 8 \( \mu \text{M} \) coated AuNPs were used for further studies for three LFA models.

Preparation of LFAs
The components of the LFAs are sample pad, conjugate pad, nitrocellulose membrane and absorbent pad. The design of strip assay was manually performed according to our previous study (26). Two different cellulose membranes having different flow rates were used in this study (M170-M120). In short, sample pads were treated with two different buffers called as buffer 4 (0.05 M Tris–HCl, 0.25% Triton X-100, 0.15 M NaCl, pH 8.0) and buffer 5 (PBS, 0.1 M NaCl, 0.2 % Tween 20), separately and dried at 37 \( ^\circ \text{C} \) or room temperature. Conjugate pads were soaked with AuNPs/Probe conjugate and dried at 37 \( ^\circ \text{C} \) for 1 h. Buffer 14 (20 mM Tris, 50 mM NaCl, 5 mM KCl, 5 mM MgCl2, 2 mM CaCl2, 0.1 mM BSA, 1.75 Triton X-100, pH 8.0), PBS and saline-sodium citrate (SSC) was used as running buffer for optimizing the assay. Test and control lines are prepared by the principle of streptavidin-biotin interaction. Briefly, biotinylated oligonucleotides were conjugated to streptavidin and then immobilized on the cellulose membrane using micropipette manually. For the assay development three sandwich models (1A, 1B, and 1.2) were prepared for hybridization on LFA and experienced separately. Two of them (1A and 1B) were for 72 base long 2019-nCoV_N3 and the last one (1.2) was for 50 base long which was obtained from shortening the 2019-nCoV_N3 region which is still specific for SARS-CoV-2.

Agarose gel electrophoresis of 2019-nCoV_N3 PCR
PCR was performed by using plasmid DNA including N gene and primers specific for 2019-nCoV_N3 were used for amplification of 72 bp...
target. This was made for verification of presenting the target gene in our sample and mimicking the real PCR samples coming from the patients, which will be applied to the LFAs for further studies. PCR products for 2019-nCoV_N3 (72 bp) are shown in Fig. S4.

**Application of targets to the LFAs models** Here probe concentration on AuNPs, sandwich models, membrane types, limit of detection (LOD) of target gene and buffer efficiency for molecular detection of 2019-nCoV_N3 on designed LFAs were studied. LFAs were prepared by four membranes and target was used as synthetic oligonucleotide sequence of 2019-nCoV_N3. Initially, buffer optimizations were experienced. For this purpose, sample pads were soaked with two buffers, buffer 4 and buffer 5, and three different buffers, buffer 14, PBS and SSC were used as running buffer. Under these conditions, the LFA control line should always appear red, and both the test line and the control line should appear red in order to say that the test is positive. Results verified that membrane type and designed models have significant differences when they are used with different buffers and temperatures (data not shown). For instance, model 1B has shown the best results with M170 membrane compared to the M120 membrane for using 4 μM and 8 μM probe concentrations at 37 °C drying (Fig. 1B, strips 1–10 and D, strips 1–4), while the model 1A has weak test lines with M170 and M120 membranes at the same temperature (Fig. 1A, strips 1–10 and C, strips 1–4). Although there are no significant differences between buffer 4 and 5 for assay results, SSC buffer was used for further studies as it has clear red color intensity on both the test and control lines and showed no nonspecific bindings on LFAs for all models using 4 and 8 μM probe concentrations. This finding is meaningful as SSC buffer has commonly positive effect on oligonucleotide hybridizations. To highlight, all developed strips have selectively detected the target and showed no nonspecific bindings to the Mers CoV_N2, Mers CoV_N3 and RdRp/Orf1 sequences of SARS CoV-2, which are specific for Mers CoV and SARS-CoV-2, respectively. This means that the designed LFA strips are suitable and reliable for molecular detection of SARS-CoV-2. Additionally, all strip assays worked truly since all the control lines are visible even if two of them are weak in positive assay (strip 1 in Fig. 1A and C). This is probably caused by the weak interaction between the capture and detection reagent for probe 1A.

**Application of PCR products to LFAs designed by three models** PCR products were applied to the developed LFAs using three models and two type of membranes. To make sure that for selective detection of target, random oligonucleotide sequences such as Se20_60 bio, Crn2SH and Mers CoV_N2, Mers CoV_N3 and RdRp/Orf1 of SARS-CoV-2, SSC running buffer were used as negative controls. Findings showed that all models recognized the target sequence, selectively without any nonspecific bindings of negative controls (Fig. 2). Although there is no significant differences between the strip assays, model 1B on both membranes might be considered as the best for the detection of 2019-nCoV_N3 in PCR sample by two probe concentrations (Fig. 2, strips 6, 8, 11, and 16). Although the test line intensity on model 1.2 (Fig. 2A, strip 1) and model 1A (Fig. 2B, strips 14 and 15) is weak compared to model 1B, they could make selective detection without any nonspecific binding to the negative controls (Figs. 1A and C and Fig. 2A, strips 2–5).
Briefly, all these results mean that designed assay models could be good candidates for naked-eye analysis of SARS-CoV-2 without advanced rRT-PCR devices and agarose gel electrophoresis. In addition, the developed LFA is considered advantageous and more sensitive than conventional agarose gel electrophoresis because it carries a very small amount of PCR products (5 μL). This is because agarose gel electrophoresis requires a large amount of PCR product and a long analysis time. There was no significant difference in the detection of PCR products between the two probe concentrations (4 μM–8 μM).

**Limit of detection of LFAs designed by three models** LOD experiments were performed by using synthetic target with developed LFAs by three sandwich models and two membrane types. It is clearly seen that model 1A works efficiently since the control lines of all strips are seen and the results showed that 0.1 μM target (72 base long) was sensitively recognized by model 1A using both membrane types (Fig. 3B, strip 3 and D, strips 1 and 3). Interestingly, there is significant difference in terms of the detection of this amount by M120 membrane. The line intensities of 0.5 μM target (Fig. 3C) are weak compared to the 0.1 μM target (Fig. 3D).

It might be said that the sensitivity of LFAs based on hybridization is significantly affected by the amount of target and membrane type. It means that there is an optimum concentration between the capture and detection oligonucleotides for effective hybridization and it is not directly related with high amount of target for this model designed with this membrane. However, the line intensities on M170 membrane were gradually become weak when the concentration of target was decreased for two probe concentrations (Fig. 3A and B). While the LOD is 0.1 μM target by using 8 μM probe (Fig. 3B, strip 3), it was 0.5 μM using 4 μM probe on this membrane. These findings suggested that probe concentrations on AuNPs have significant role for sensitive detection along with the membrane type. This can also be verified by comparing the strips developed by 4 μM probes with M170 (Fig. 3B, strip 1) and M120 membrane (Fig. 3D, strip 1) for the same LOD.

LOD experiments were experienced by model 1B using both membranes and two probe concentrations (Fig. 4). According to the results, 0.1 μM target (72 base long) was detected by M170 membrane using both probe concentrations (Fig. 4B) while it was 0.005 μM by M120 membrane using 8 μM probe (Fig. 4F). These results verified that membrane types and probe concentrations used in LFAs have significant role for sensitive detection. Here, the sandwich model, another important aspect of high-sensitivity detection, appears to be superior to model 1A, as all strips have clear line intensities and low detection limits.

LOD was also experienced by model 1.2 using both membranes and two probe concentrations (Fig. 5). According to the results the minimum amount of target, 50 base long, was detected as 5 pM by M170 membrane using 8 μM probe (Fig. 5C, strip 1) while it was 100 pM by M120 membrane using 8 μM probe concentration (Fig. 5E, strip 3) without any nonspecific bindings. This amount is either lower than the reported nucleic acid based LFAs (28,29) or similar with the amount of SARS-CoV-2 N protein detection (30). Thus, to make sensitive recognition, 8 μM probe could be used for this model. Lastly, model 1.2 allowed effective hybridization on both lines and this was resulted by clear line intensity and the lowest detection amount of target.

When compared to all models in terms of the LOD, the length of the target has crucial role for LFA efficiency. It could be inferred from these results 50 base long target sequence could be sensitively recognized compared to the 72 base long as the base length of target sequence become shorter, LOD was observed as lower. This may be caused by the high probability of the hairpin structures in long bases, which can interfere the hybridization on the assay. Therefore, sensitive detection is closely related with the length of target sequence and hybridization models between the target and capture reagents for LFA. Along with this, it should be highlighted that the main gene length (72 base long) is also clearly detected by developed strip assay models and could be used for the detection of 2019-nCoV_N3.

Lastly, LFAs were also prepared by different times in order to see the stability of conjugates and LFAs efficiency. Therefore, assays were applied by 6 months awaited conjugates. It was found that conjugates were still stable and worked efficiently in terms of the hybridization on the LFAs and both lines on the strips were clearly observed (data not shown). Since the stability of the conjugates is also highly related with the red color of suspension, they still have their original color (data not shown). Therefore, designed LFAs with these models have potential for long shelf life if they are fabricated. Because the developed method is also consistent in terms of the reproducibility and there was no difference in batch to batch production or preparation of all LFA strips.

![Figure 3](image.png)

**FIG. 3.** LOD of target by model 1A using M170 (A, B) and M120 (C–E). (A) 0.5 μM, (B) 0.1 μM, (C) 0.5 μM, (D) 0.1 μM, (E) 0.005 μM target. Strips 1 and 2 were prepared by 4 μM probe and strips 3 and 4 were by 8 μM. Strip E1 was prepared by 4 μM and E2 was by 8 μM, and then target was applied to both. Strips 1–3 were all applied by target and strips 2–4 were by buffer as a negative control. Arrows show the test and control lines.
As a conclusion, the detection of SARS-CoV-2 by targeting the 2019-nCoV_N3 gene region was succeeded by designed LFAs models as a first study according to the best of our knowledge. Although the detection of virus by LFAs is commonly based on the immunoglobulins of patients or antigens, these models are for the molecular detection of SARS-CoV-2 since it is the most reliable method in the world. LFA is cost-effective because it can be analyzed by the naked eye, allowing conventional PCR products to be used instead of expensive analyzers and reagents such as rRT-PCR. We believe that findings will be valuable for various molecular detection methods for SARS-CoV-2 and its mutants. Because assay is based on the hybridization and can be rapidly designed for specific sequences of mutant viruses. Thus, the detection of either mutated or conserved regions could be possible by these type of assay models. Since the PCR products were recognized and parameters were optimized in designed LFAs they might also be a candidate for point of care diagnosis in terms of the molecular detection of SARS-CoV-2 for further fabrication. In this perspective, applying the developed LFAs to the real samples coming from the patients will be planned for the future work.

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