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# An enzymatic paper-based biosensor for ultrasensitive detection of DNA

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## 1. ABSTRACT

Point-of-care Nucleic acid testing (POCNAT) has become an attractive technique for DNA identification in resource-limited settings, offering a rapid system for urgent clinical applications. In this study, a chemiluminescence-based lateral flow biosensor (CL-LFB) was developed for the quantitative analysis of DNA, without labeling and amplification. The developed biosensor employs a two-step hybridization, a primary hybridization of 5´-biotinylated detector probe to the target DNA and a secondary hybridization of the resulting complex with the immobilized capture probe. Quantitative analysis of DNA was provided via HRP-catalyzed reaction with the chemiluminescense substrate followed by imaging with a complementary metal-oxide-semiconductor (CMOS) digital camera. The assay performance was investigated using a synthetic target, *16S rRNA* gene (775 bp) and the whole genome derived from *Escherichia coli (E.coli).*

A detection limit of 1.5 pM for the synthetic target and 0.4 ng/ml for *16S rRNA* gene was obtained. In spite of LFBs limitations for the detection of large DNA fragments, the proposed assay provided a low-cost, fast, and sensitive tool for PCR-free diagnosis of small and larger fragments of nucleic acids.

## 2. INTRODUCTION

In recent years nucleic acid detection has become a diagnostic necessity in food safety analysis, veterinary, environmental monitoring, forensics, biological research, and modern medical diagnostics (1). Current nucleic acid testing methods are mostly on the basis of polymerase chain reaction (PCR) and still require specialized personnel and complicated instruments (2). Consequently, there is a large demand for cost-effective, easily operated, sensitive and quantitative diagnostic devices for the detection of nucleic acids (3). Meeting this requirement, there is a large number of point of care nucleic acid testing (POCNAT) devices that have been developed for fast and sensitive detection of DNA and RNA (4). Among the various types of POCNATs, nucleic acid lateral flow biosensor (NALFB) has attracted a growing interest in clinical molecular diagnostics (4). The major advantages of these paper based devices are cost–effectiveness, rapidness, simplicity, portability and its potential to be employed in the absence of skilled personnel and expensive instruments (5). To obtain quantitative information from paper- based diagnostic devices, various approaches based on fluorescent nanoparticles including quantum dots and up-converting phosphors (6-8), enzyme amplification (9-11), Surface-Enhanced Raman Scattering (SERS) (12, 13), and electrochemical evaluation (14) have been developed. In order to provide quantitative bio-sensing, a number of portable strip readers are designed based on the type of label employed in the assay (15).

Among the mentioned labels, enzymes are one of the most studied reporters in the development of bioassays and biosensors (9, 16-19). The most frequently used enzymatic label is horseradish peroxidase (HRP), however alkaline phosphatase (20), cholinesterase and glucose oxidase were also employed (9, 21). Meanwhile, assay sensitivity can be enhanced by loading enzymes on gold nanoparticle (9, 10, 22) and carbon nanotubes (23). Chemiluminescence (CL) detection of enzymatic labels has also been an ideal diagnostic method in a number of commercial point of care devices (24-28). The photon emission due to enzyme-catalyzed CL can be detected by photomultiplier-based and light imaging instruments based on charge-coupled-device (CCD) or complementary metal-oxide semiconductor (CMOS) sensor (24, 28). This approach has demonstrated a great potential to be employed as a reliable analytical tool, due to its sensitivity, short assay time and less reagent consumption (28).

Prokaryotic cells have always been ideal for research in molecular biology and biochemistry. The most commonly used species of bacteria is *Escherichia coli* (*E.coli)* because it is easily propagated in the laboratory, grows rapidly under well-defined laboratory conditions, and its entire genome sequence has been determined (29). Bacterial *16S ribosomal RNA* (rRNA) gene, comprising species-specific sequences, has been widely used for quantification, taxonomic studies and identification of bacterial species (30). There have been lots of reports on the application of *16S rRNA* gene for the detection of pathogens by means of biosensors (30, 31).

In the current study, a CL-based nucleic acid lateral flow biosensor (CL-NALFB) has been developed to detect DNA sequences, without labeling or amplification. The proposed biosensor takes advantage of lateral flow simplicity, rapidness, and POC application in addition to quantitative analysis of the CL signal. This technique was established on the basis of “sandwich hybridization” strategy in which target DNA is hybridized to the capture and detector probes. Streptavidin-labeled Horseradish peroxidase (StAv-HRP) was employed as label and the resulting CL signal on the reaction zone was generated by the HRP-catalyzed reaction with luminol/$H_2O_2$ substrate. Subsequently, CL images were acquired using a conventional CMOS digital camera at ambient temperature. The presented CL-NALFB was first optimized with a 50 nt synthetic target. However, it also exhibited satisfactory results when *16S rRNA* gene (775 bp) and *E.coli* ATCC 25922 genome were used to evaluate the operation of this system for PCR-free detection of larger fragments. This system confirms the feasibility of CL-NALFB strategy for quantitative detection of DNA, and it can be implemented in the diagnosis of different infectious agents, genetic disorders, small RNAs and etc.

## 3. MATERIALS AND METHODS

### 3.1. Materials

#### 3.1.1. Chemicals and apparatus

Bovine serum albumin, Tween 20, Tris- Base, Tris- HCl, sodium chloride, sodium citrate, yeast extract, peptone, agar, phosphate-buffered saline, enhanced CL substrate (ECL) and polyclonal anti-streptavidin (anti-StAv) antibody were purchased from Sigma-Aldrich Co. (MO, USA). HRP- StAv conjugate was purchased from R&D, Inc. (Minneapolis, MN). PCR Master Mix was purchased from Ampliqon (Odense, Denmark). All chemicals were of analytical grade and were used without further purification. Cellulose

**Table 1.** Sequences used for chemiluminescence-based NALFB development

| Function | Length | End-label | Nucleotide sequence (5'-3') |
|---|---|---|---|
| Synthetic target | 50 | None | AAAAAAAAAAAAAAAAAAAAAAAAATTTTTTTTTTTTTTTTTTTTTTTT |
| Capture | 20 | None | TTTTTTTTTTTTTTTTTTT |
| Detector | 20 | Biotin | AAAAAAAAAAAAAAAAAAA |
| Non-complementary DNA | 50 | None | CGACGAGGACCGACCACGCACGGAAGCGCGTCGCGTTGCGCTGGGCTCTT |
| Escherichia coli 16S rRNA | 775 | None | GenBank: X80724.1 |
| Capture | 35 | None | CTGAAAGTACTTTACAACCCGAAGGCCTTCTTCAT |
| Detector | 35 | Biotin | GTCAATGAGCAAAGGTATTAACTTTACTCCCTTCC |
| Forward primer | 20 | None | TGCCTGATGGAGGGGGATAA |
| Reverse primer | 19 | None | TTTAACCTTGCGGCCGTAC |

fiber sheets (CFSP223000) and nitrocellulose membrane (HFB24004) were obtained from Millipore (Billerica, MA). Laminated cards were purchased from Rojan Azma Co. (Tehran, Iran). *Escherichia coli ATCC 25922* (American Type Culture Collection), *Klebsiella pneumonia* ATCC13883, *Enterococcus faecalis* ATCC19433, *Proteus mirabilis* ATCC4371, *Serratia marcescens* ATCC13880, *Staphylococcus haemolyticus* ATCC43252 and *E.coli* PTCC1276 was obtained from the department of medical bacteriology, Tarbiat Modares University. The CL images were acquired with a bench-top dark box equipped with a professional CMOS digital camera (Canon G12, Canon Inc., Tokyo, Japan).

### 3.1.2. Oligonucleotides

The oligonucleotide probes, primers and targets are listed in Table 1. The capture and detector probe sequences were designed by Liao *et al* (31). All synthetic oligonucleotides were purchased from Fazabiotech, Inc. (Tehran, Iran) and dissolved in sterile distilled water to 100 µM concentration. All solutions and buffers were prepared with ultrapure (greater than 18 MΩ) water from the Millipore Milli-Q water purification system (Billerica, MA).

## 3.2. Methods

### 3.2.1. Preparation of the NALFB

The NALFB was constructed using sample pad, nitrocellulose membrane and absorbent pad. All of the compartments were laminated manually with a 2mm overlap. The sample application pad (17 mm ×4mm), was used without further saturation. Nitrocellulose membrane (25 mm ×4 mm) was first saturated with 20X saline-sodium citrate (SSC) buffer (PH=7.7) for 10 minutes and then dried at 37 °C. Test zone was prepared by immobilizing 1 µl of capture probe solution with the concentration of 2 µM, then the membrane was baked at 80° C for 1 hour. Subsequently, Anti-StAv antibody was diluted to 0.5. mg/ml and was manually coated on the control zone. The distance between test and control areas was about 7mm.The membrane was dried for 2 hours at 37 °C and blocked in 1X Tris-buffered saline (TBS), PH= 7.4, containing 0.05 percent Tween 20 and 1 percent BSA. The nitrocellulose membrane was then dried at 37°C for 2 hours and stored at 4 °C. The nitrocellulose membrane was first pasted to the backing card, then sample and absorbent pads were laminated sequentially. All layers were laminated with appropriate overlap to ensure a constant flow of sample solution. The whole assembled strip was about 4mm in width. The strips were stored at 4° C in a sealed bag comprising desiccant bags.

### 3.2.2. Culture conditions

Frozen stock of *E.coli (*ATCC 25922), *Klebsiella pneumonia* ATCC13883, *Enterococcus faecalis* ATCC19433, *Proteus mirabilis* ATCC4371, *Serratia marcescens* ATCC13880 ,*Staphylococcus haemolyticus* ATCC43252 and *E.coli* (PTCC 1276) were plated on LB agar (yeast, 5gr/L; peptone, 10gr/L; NaCl, 10gr/L; agar, 15gr/mL; pH 7.3) and incubated overnight. A single colony was used to seed LB broth media (yeast extract, 5 gr/L; peptone, 10 gr/L; NaCl, 10 gr/L; pH 7.3) overnight. The viable cell count of all bacterial strains was determined by surface plating of 100 µL of the prepared dilutions ($10^1$-$10^8$) on LB plates. The bacterial suspensions were serially diluted by 10-fold in PBS buffer.

### 3.2.3. DNA extraction and PCR assay

The genomic DNA of all bacteria was extracted from the overnight culture by G-spin™ Total DNA Extraction Mini Kit (Intron, Seoul), according to manufacturer's instructions. The extracted genomes were stored at -20 °C until required.

The listed primers in Table 1 were used to amplify a fragment of 775 bp from *E.coli 16S rRNA* gene. On the basis of *in-silico* analysis, the 775 bp

sequence is conserved within all *E.coli* genomic DNA sequences in NCBI. PCR was performed with the following conditions: 1 µL of each primer (10 µM), 1 µL of extracted DNA, 6.25 µL of Ampliqon Master Mix and 3.25 µL of ultra-pure water. The thermal cycling condition was as follows: initial denaturation at 95 °C for 10 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 64 °C for 30 s and extension at 72 °C for 50 s, furthermore a final extension at 72° C was considered for 10 min. The PCR product was electrophoresed in 1 percent agarose gel containing ethidium bromide (1 mg/ml) and TAE buffer (PH=8.3). The 775 bp products were visualized under UV light.

#### 3.2.4. Assay procedure

HRP- StAv conjugate (1:200 v/v) and the synthetic target (with the final concentration ranging from 10 pM to 10 nM) were diluted in 100 µL of 2X SSC buffer (PH=7.7). Detector probe was added to the final concentration of 0.5 µM. The resulting solution was thoroughly mixed and incubated at room temperature for 15 minutes. Then, 100 µL of the prepared sample solution was dropped onto the sample pad. After 10 to 15 min, the biosensor was washed by adding 50 µL of the running buffer (SSC 2X, PH=7.7) on the sample pad.

For identification of the *16S rRNA* gene and *E.coli* genomic DNA, the target DNA was first heat-denatured in a thermocycler (95 °C) for 5 min and immediately chilled in ice to obtain single-stranded DNA. The NALFB procedure was almost the same as synthetic target, but with a slight modification. Different concentrations of 16S rRNA gene (2.6 µg/ml to 0.04 µg/ml) and *E.coli* genomic DNA ($24\times10^4$ to 12 ng/ml) were carefully prepared in milli-Q water. Then, 10 µL of each concentration was added to 90 µL of SSC 4X (PH=7.7) containing the detector probe (0.5 µM). The resulting hybridization solution (100 µL) was incubated at 60°C for 15 min to allow target-detector probe hybridization. The sample solution was added to the NALFB and allowed to migrate. Finally, 50 µL of SSC 4X (PH=7.7) containing HRP-StAv (1:200) was dropped onto the sample application space of the NALFB. The NALFB was subsequently washed by adding 20 µL of SSC 4X (PH=7.7). The CL signals were generated by applying 20 µL of enhanced CL substrate directly to the detection zone and the signals were acquired using a bench-top dark box equipped with a professional CMOS digital camera. The integration time was adjusted to 30 s in order to take long exposure images, resulting in pictures with the highest feasible level of CL signal. In the quantification step, CL signals were elaborated using ImageJ 1.51n, free image processing software (ImageJ, National Institute of Health, Bethesda, Maryland, USA).

## 4. RESULTS AND DISCUSSION

### 4.1. Design strategy of the CL-NALFB

The assay principle of the CL-based LFB is shown in Figure 1-2. In the presence of the target DNA two circular CL signals were observed, however in the absence of target only one signal corresponding to the control zone was detected. If no signal is detected, the CL-NALFB is not working properly. Quantitative analysis was performed by measuring the mean photon emission of test and control zones using ImageJ 1.51n software. The T/C ratio was used for the measurements to increase the accuracy of the method by counteracting the background and inherent heterogeneity of the test strip (32, 33). To analyze the amount of DNA quantitatively, DNA standard solutions were applied to the biosensor and the calibration curve was generated by plotting the T/C ratio against the logarithm of the target concentration.

### 4.2. Optimization of assay parameters for the synthetic oligonucleotides

Different analytical parameters, including the concentration of detector and capture probes, running buffer and signal acquisition time were optimized to obtain the best performance. To evaluate the optimal conditions, only the variations of CL signal intensity corresponding to test zone was determined (28).

#### 4.2.1. The optimal concentration of capture and detector probes

The detector and capture oligonucleotide probes were used with the final concentration of 0.5 and 2 µM. The assay conditions were first optimized by variation in the amount of biotinylated detector probe within the hybridization solution. Concentration of detector probe was optimized to create an assay with a suitable limit of detection (LOD). As a general rule, hybridization rate and sensitivity increases with the probe concentration. To evaluate the optimal concentration of the detector probe, we determined CL signal intensity of the test zone and its variations in the presence of different concentrations of detector probe (0.1 µM to 1 µM) and a fixed amount of target DNA. As shown in Figure 3B, CL intensity of the test zone increases upon raising the detector probe concentration from 0.1 to 0.5 µM, however further increase of the detector concentration did not have a significant effect on the signal intensity. Therefore, 0.5 µM was selected for the subsequent experiments.

Optimization of the capture probe concentration is also crucial for quantitative analysis of the target DNA and it greatly influences the performance of NALFB. The capture probe

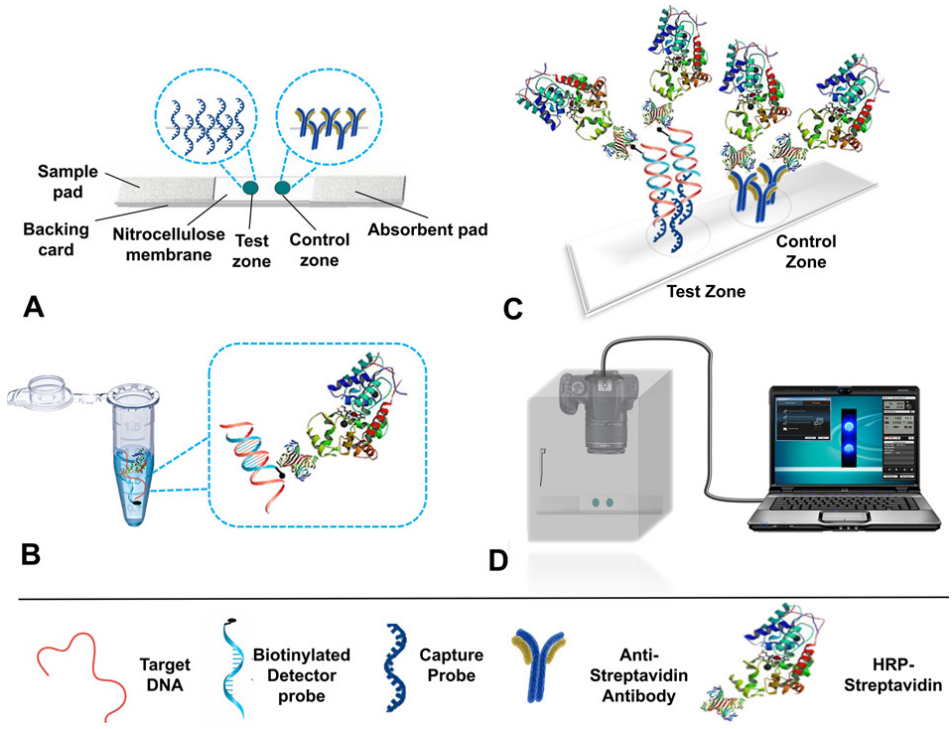


**Figure 1.** Schematic illustration of the synthetic DNA identification based on nucleic acid lateral flow biosensor and chemiluminescent (CL) analysis. A) Schematic illustration of the CL-NALFB configuration for quantitative assessment of target DNA. B) The detector probe, target DNA and HRP- StAv conjugate were mixed to generate the HRP conjugate-detector probe-target DNA complexes. C) The HRP label-detector probe-target DNA complexes were captured by the oligonucleotide capture probes on the test zone and the excess HRP-Streptavidin conjugates were captured by Anti-Streptavidin antibody on the control zone. D) CL detection using a bench-top dark box equipped with a professional digital camera.

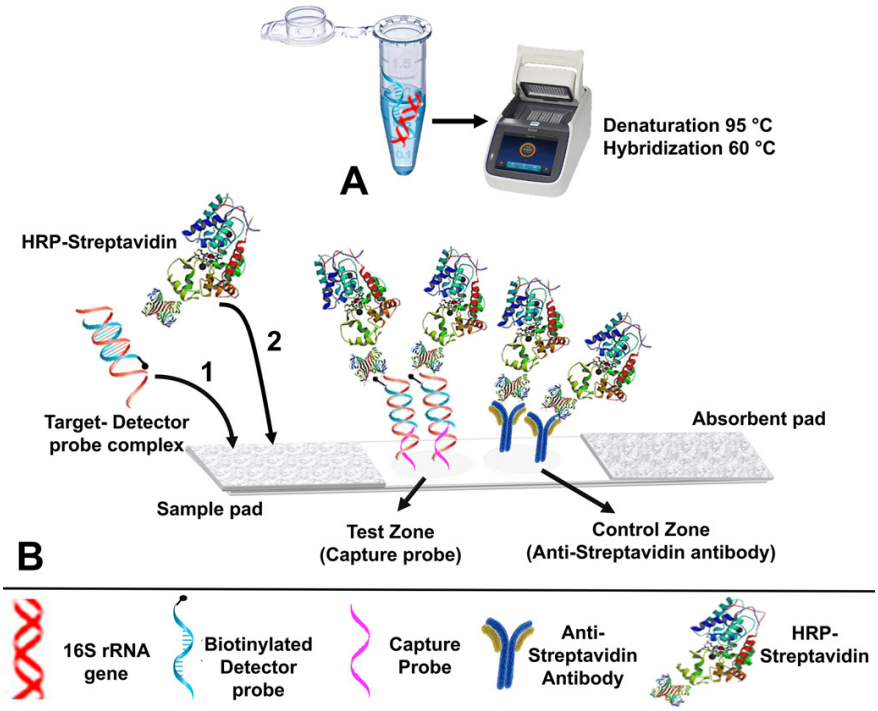


**Figure 2.** Schematic representation of 16S rRNA gene detection using CL-NALFB. A) In the first step, the target gene was denatured and hybridized to the detector probe. B) The 16S rRNA-detector probe complex was applied to the biosensor (1), subsequently, HRP-StAv label was added (2) and the NALFB was visualized using a professional camera.

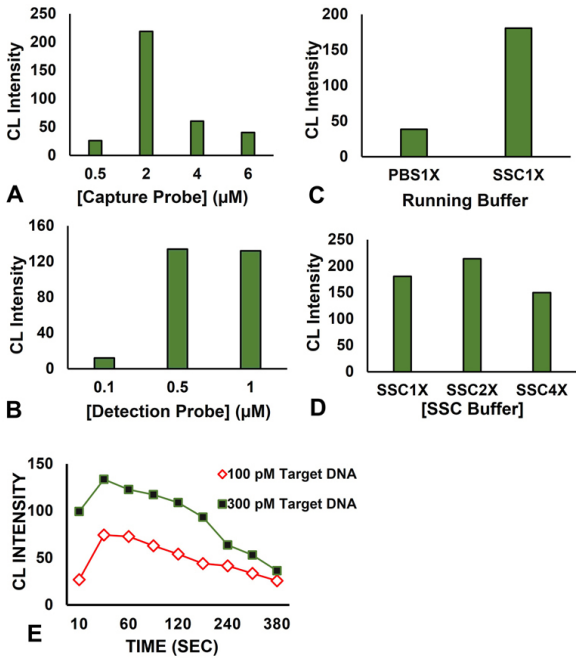


**Figure 3.** Optimization if the assay parameters. A) Effect of the capture probe concentration on the test zone chemiluminescence (CL) intensity. B) Effect of detector probe concentration on the test zone CL intensity. C) Effect of running buffer on the test zone CL intensity. D) Effect of SSC buffer concentration on the test zone CL intensity.

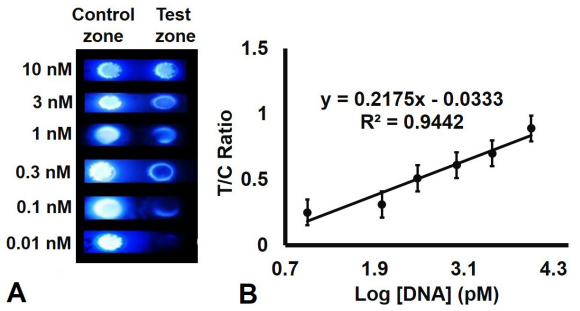


**Figure 4.** A) CL images of the developed CL-NALFBs acquired with the digital camera after applying the standard solutions ranging from 0.01 to 10 nM. B) The calibration curve of CL-NALFB for the detection of target DNA.

optimization was accomplished using a fixed amount of synthetic target DNA. The CL signal corresponding to the test zone increased by raising capture probe concentration from 0.5 to 2 μM, however further increase of the capture probe concentration decreased the signal considerably. This result may be interpreted as due to massive probe accumulation and overlapping (34), resulting in lower availability of the probes and therefore less hybridization. As a result, the optimized capture probe concentration of 2 μM was used in the subsequent experiments (Figure 3A).

### 4.2.2. Optimization of the running buffer solution

The performance of NALFB and the hybridization efficiency are significantly affected by running buffer solution (12). In the current study, PBS1X (PH=7.4.) and SSC1X (PH=7.7) buffers were tested as running buffer solutions. The CL signal of the NALFB using SSC1X buffer was notably higher than that of PBS running buffer (Figure 3C), therefore SSC buffer was selected for subsequent experiments. Additionally, SSC buffer concentration affects the performance of NALFB by influencing hybridization temperature, specificity and stringency (35). Hereof, different SSC buffer concentrations ranging from 1X to 4X were examined (Figure 3D). The CL signal decreased with 4X SSC running buffer, however it was expected that there would be an increase in the DNA hybridization and signal intensity due to low stringency conditions. This may be because AT-rich base pairs are less stable in comparison to GC rich sequences in the solutions containing higher concentration of NaCl (35). Therefore, 2X SSC buffer was used as running buffer in all experiments.

### 4.2.3. Optimization of the signal acquisition time

The signal acquisition time was optimized by measuring the absolute CL intensity of the test zone at different integration times (10 s to 6.33 min). It was observed that 30 s was sufficient to obtain the maximum CL signal intensity. As shown in Figure 3E, using an integration time lower than 30 s exhibited a weak CL signal, while the integration times higher than 30 s did not enhance the CL signal. The subsequent decline of the CL signal is possibly due to partial inactivation of HRP in the presence of intense CL signal (25).

## 4.3. Analytical performance of CL-NALFB using synthetic target

For precise calculation of DNA concentration in the unknown samples and to investigate the ability of the proposed assay to quantitate target DNA, a calibration curve was constructed using 6 standard solutions with the DNA amount ranging from 10 pM to 10 nM. Figure 4A shows the images of CL-NALFBs after applying different concentrations of the target DNA. The CL signal intensity is directly proportional to the amount of sandwich complex formed on the test zone.

The T-zone/C-zone ratio was plotted as a function of the DNA concentration logarithm to generate a calibration curve with a coefficient of determination of 0.9442 and regression equation of $y = 0.2175x - 0.0333$ (Figure 4B), the obtained calibration curve was linear over the 0.01-10 nM range. The above equation would be applicable for estimating analyte concentration in the unknown samples. The reproducibility of the assay

**Table 2.** Comparison of the proposed chemiluminescence-based NALFB with the other reported LFBs for nucleic acid detection

| Brief Assay Strategy | Test Zone[1] | LOD | Response Time (min) | Reference |
|---|---|---|---|---|
| Lateral flow biosensor based on DNA sandwich hybridization/ Using carbon nanotube-detection probe as label/Quantitative detection using imageJ software | StAv[2] –biotinylated capture probe | 40 pM | NA[3] | (44) |
| Using AuNP-Det[4] probe as label /Detection based on sandwich hybridization/ quantification by GoldBio strip reader | StAv-biotinylated capture probe | 0.5 nM | 15 | (45) |
| Using fluorescent carbon nanoparticle-Det probe as label/ Based on sandwich hybridization/Quantitative detection by ESE-Quant Lateral Flow Reader | StAv-biotinylated capture probe | 0.4 fM | 20 | (46) |
| Using Latex Bead-Det probe as label /quantification by Gold Bio strip reader/ Based on sandwich hybridization | StAv-biotinylated capture probe | 3.75 fmol<br>=28.85 pM | 20 | (47) |
| Using AuNP-Det probe as label/ Based on sandwich hybridization /Quantification by Surface Enhanced Raman Scattering | Capture probe | 0.24 pg/ml | NA | (12) |
| Using AuNP-Det probe as label/Based on sandwich hybridization/ visual detection | Capture probe | Less than 50 fmol | NA | (48) |
| Based on Isothermal Strand Displacement Polymerase Reaction(ISPDR) and gold nanoparticles | Capture probe | 0.01 fM | 30 | (49) |
| Using HRP-Streptavidin as label/ based on CL[5] detection/ light imaging by digital camera/ Quantitative detection using imageJ software | Capture probe | 1.5 pM<br>=150 attomole | 30 | This work |

[1]The immobilized reagent on the test zone, [2]Streptavidin, [3]Not available, [4]Detector, [5]Chemiluminescenc

was determined by analyzing the NALFBs, in triplicate (36), in the presence of 10 nM and 0.25 nM target DNA. The coefficient of variation (CV percent) was found to be 1.1 percent and 5.6 percent, respectively. The detection limit was determined to be 1.5 pM, using the IUPAC standard method ($LOD=y_{Blank}+3SD_{Blank}$), $SD_{Blank}$ is the standard deviation of blank measurements and $y_{Blank}$ is the concentration corresponding to T/C ratio of the blank (12).

The LOD, response time and assay strategy were compared to that of previously published papers on NALFBs (Table 2). As shown in Table 2, the DNA biosensor in the present work is notably comparable to the biosensors using gold nanoparticle (GNP) and latex beads as label. The detection limit of the reported CL-NALFB is approximately 330 times lower than that of GNP-based NALFBs without signal amplification and 40 times lower than the detection limit of carbon nanotube-based NALFB. Another important advantage of the proposed CL-based biosensor is its convenient preparation in comparison to carbon nanoparticle, latex bead and GNP-based NALFBs mentioned in Table 2.

#### 4.3.1. Evaluation of cross-reactivity

The selectivity of CL-NALFB was evaluated in the presence of 20 nM target DNA, 0 nM target DNA and 20 nM non-complementary DNA (Table 1) under the same conditions. It was found that signal intensity in the presence of non-complementary strand was almost the same as blank, however, in the presence of target DNA with the same concentration, a remarkable increase in the signal intensity was observed (Figure 5). The results demonstrated that the CL-NALFB was able to differentiate non-complementary DNA from the target DNA.

### 4.4. Optimization of assay parameters for *16S rRNA* gene

To obtain the best performance of the CL-NALFB for identification of *16S rRNA* gene, the key parameters including, the concentration of running buffer and the hybridization temperature were optimized. In order to evaluate the optimized condition, the variations in CL signal of the test zone was determined. In addition, we took advantage of prior optimizations on the synthetic target.

#### 4.4.1. The optimal running buffer solution

We noticed that SSC buffer concentration remarkably affected the signal intensity of test zone (Figure 6A). It can be seen that the signal intensity increased with the increase in SSC concentration from 2X to 4X, while a higher concentration did not significantly increase the signal intensity. Therefore, 4X was sufficient to achieve maximum intensity.

#### 4.4.2. Optimization of the hybridization temperature

The effect of temperature on DNA hybridization was investigated by analyzing the CL signal corresponding to test zone at different

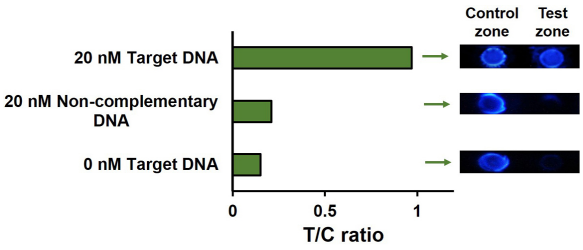


**Figure 5.** Typical images and T/C ratios corresponding to CL-NALFBs in the absence and presence of 20 nM target DNA and 20 nM non-complementary DNA.

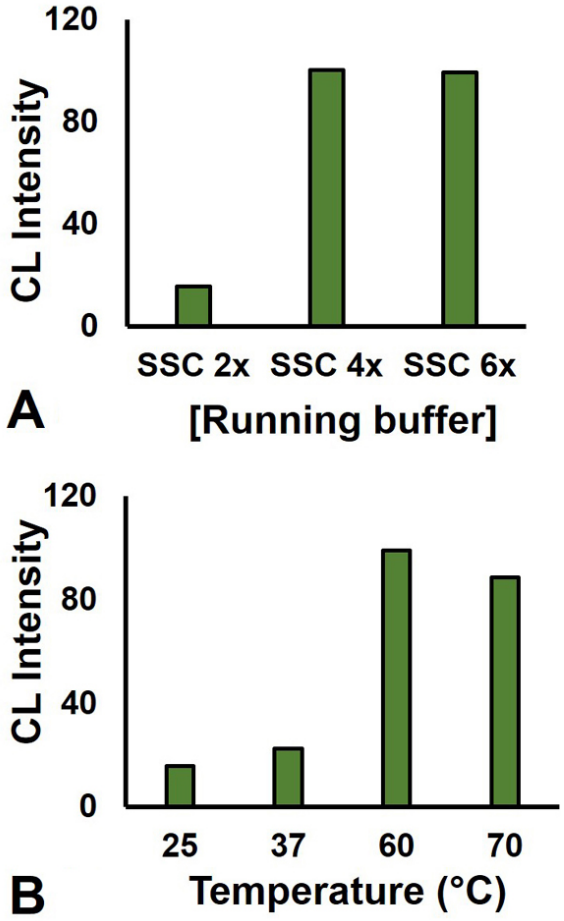


**Figure 6.** A) Effect of running buffer on the CL intensity of test zone. B) Effect of hybridization temperature on the test zone CL intensity. Target DNA concentration: 60 ng/ml

hybridization temperatures. The hybridization temperature remarkably affects the binding behavior of nucleotide strands. Hybridization bellow the optimal temperature increases cross-hybridization, while, hybridization above this temperature may result in reduced signal intensities (37).The parameters affecting the hybridization temperature include salt and formamide concentration, GC content and homology between probe and target (20). According to Kong *et al.* (38) usually 60 °C is appropriate for hybridization, and a 5°C gap between hybridization temperature ($T_h$) and melting temperature ($T_m$) is unnecessary. $T_m$ value of the detector probe was predicted to be 71.3°C using the calculator provided by Sigma–Aldrich. Therefore, 25° C, 37° C, 60° C and 70° C were tested as hybridization temperature in the first step. Figure 6B presents the effect of $T_h$ on the CL signal intensity of test zone. The highest signal intensity was obtained with 60° C, which was then used in the subsequent experiments.

### 4.5. Performance of CL-NALFB using *16S rRNA* gene

The performance of the developed paper-based DNA biosensor was investigated using the *16S rRNA* gene derived from *E.coli* (ATCC 25922) by PCR-amplification. The specificity of primers and probes for identification of *E.coli 16S rRNA* gene was previously investigated using 6 common bacteria in UTI (*Klebsiella pneumonia* ATCC13883, *Enterococcus faecalis* ATCC19433, *Proteus mirabilis* ATCC4371, *Serratia marcescens* ATCC13880, *Staphylococcus haemolyticus* ATCC43252, *Pseudomonas aeruginosa* ATCC27853) (39, 40).

In this assay, we applied the heat denatured 775 bp *16S rRNA* gene without labeling on the CL-NALFB, unlike other works that detected tagged bacterial gene amplicons (41, 42). In order to evaluate the analytical performance of the biosensor, different concentrations of the 775 bp DNA (2.6 µg/ml to 0.04 µg/ml) were prepared. Following optimization of the assay conditions, the CL signal corresponding to test and control zone, for each concentration, was analyzed using ImageJ software. By plotting the T/C ratio versus the log of DNA concentration, a calibration curve with the regression equation of y = 0.2456x + 0.12 was obtained (Figure 7). It can be seen that the signal intensity rose with the increase in the concentration of the target DNA and a good linear relationship was obtained in the range of 2.6 µg/ml to 0.04 µg/ml. On the basis of the IUPAC standard equation (as previously mentioned), the LOD was estimated to be 0.4 ng/ml. This value is equal to 4.78 × $10^7$ copies of *16S rRNA* gene in a total volume of 100 µL. Noguera *et al.* (41) fabricated a nucleic acid lateral flow immunoassay (NALFIA) based on rapid amplification with tagged primers and carbon nanoparticle as label. In this work, the genes (130-96 bp) encoding virulence factors of shiga toxin producing *E.coli* were detected. Their biosensor achieved a detection limit between 0.1 to 0.9 ng/µL. This value is approximately 1000 times less sensitive than CL-NALFB approach. In 2017, Aissa *et al.* (42) designed a NALFIA based on GNP for the detection of *E. coli*

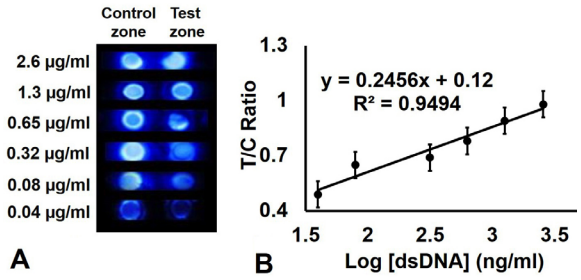


**Figure 7.** A) Images of the CL-NALFBs in the presence of different concentrations (2.6 – 0.04 µg/ml) of 16S rRNA gene. B) The resulting calibration curve.

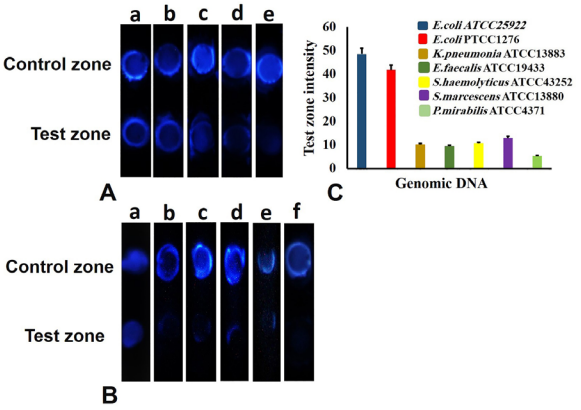


**Figure 8.** The developed CL-NALFB for the detection of *E.coli* ATCC 25922. A) CL images of the developed CL-NALFBs acquired with the digital camera after applying the standard 10-fold dilutions of genomic DNA: a-e: $25\times10^4$ – 12 ng/ml. B) Specificity of NALFB for *E.coli* detection. a-f: *E.coli* PTCC1276, *Klebsiella pneumonia* ATCC13883, *Enterococcus faecalis* ATCC19433, *Staphylococcus haemolyticus* ATCC43252 , *Serratia marcescens* ATCC13880, *Proteus mirabilis* ATCC4371. C) CL intensity of test zone in the presence of $2\times10^5$ ng/ml genomic DNA extracted from E.coli ATCC25922, *E.coli* PTCC1276 and 5 prevalent uropathogenic bacteria *Klebsiella pneumonia* ATCC13883, *Enterococcus faecalis* ATCC19433, *Staphylococcus haemolyticus* ATCC43252, *Serratia marcescens* ATCC13880, *Proteus mirabilis* ATCC4371.

*eaeA* gene (151 bp) and *Salmonella enterica yfiR* gene (375 bp), being PCR-amplified by quadruple-tagging set of primers. Their LFB achieved LOD of 34 ng/ml for the detection of *E. coli* and LOD of 95 ng/ml for *Salmonella*. Roskos *et al.* (2013), described the detection of *Mycobacterium tuberculosis* using Loop Mediated Amplification (LAMP) and Exponential Amplification Reaction (EXPAR) coupled to visual detection of the amplification products with the NALFB. They successfully detected 3 copies and $2x10^4$ copies of genomic DNA using LAMP and EXPAR coupled to NALF, respectively (43).

The assay reproducibility was evaluated in the presence of 0.08 µg/ml and 2.5 µg/ml of the *16S rRNA* gene. The NALFBs were analyzed in triplicate, and the CVs were found to be 6 percent and 3.1 percent, respectively.

### 4.6. Performance of CL-NALFB for the detection of *E.coli*

To demonstrate the feasibility of the proposed biosensor for practical applications, the CL-NALFB was applied for direct detection of *E.coli* ATCC 25922 genomic DNA without any amplification step (PCR-free) and labeling. To this end, the prepared bacterial suspension ($10^8$ CFU/ml) was subjected to genomic DNA extraction and the prepared 10-fold DNA dilutions in milli-Q water were applied to CL-NALFB. It is worth to note that *16S rRNA* gene, as the biosensor target in this study, is well characterized, consists of both variable and conserved regions and has been widely employed for molecular diagnostic purposes (31).

As shown in Figure 8A, CL intensity was directly proportional to the concentration of genomic DNA. Furthermore, the minimum concentration that could be observed using the digital camera was visually determined to be approximately 12 ng/ml, which is 30-folds higher than that of 16S rRNA gene (775 bp) detection. This reduction in sensitivity can be attributed to the large molecular weight of genomic DNA, causing a low migration rate on nitrocellulose (45). Indeed, the developed biosensor exhibited a lower LOD as compared to the NALFB proposed by Mao *et al.* (2.5 µg/ml). These investigators had successfully developed a NALFB based on GNPs and double-probe hybridization, capable of detecting human genomic DNA within 20 min (45). Very few studies on the application of NALFBs for direct detection of genomic DNA have been published. Previous studies have commonly reported the use of amplification-based approaches coupled with NALFBs for the detection of various organisms such as *Rice Black-Streaked Dwarf* virus (50), *Group B Streptococcus*(51), microRNA 21 and let-7a (52), foot-and-mouth disease virus (53), *Influenza A virus* (54), *Toxoplasma gondii* (55,56) and *Macrophomina phaseolina* (57). PCR-based approaches, while powerful, are usually time- and labor-intensive, requiring complex and expensive instruments and qualified personnel (58). Recently, Li *et al*. reported the development of a NALFB coupled with immunomagnetic separation and asymmetric PCR for the detection of *Lysteria monocytogenes*. Using this approach, a detection limit of $3.5\times10^4$ CFU/g in PBS buffer was obtained, while, the whole assay procedure was completed in 6 h (59).

In recent years, direct detection of genomic DNA using biosensor technology has attracted considerable interests and has shown promising results. In 2018, Li *et al.* developed a novel electrochemical biosensor based on multi signal probes (MSP) system to directly detect *mecA* gene on *methicillin*-resistant *Staphylococcus aureus* (MRSA) genome. This genosensor successfully detected 57 fM MRSA genomic DNA (60). In another study, Leonardi

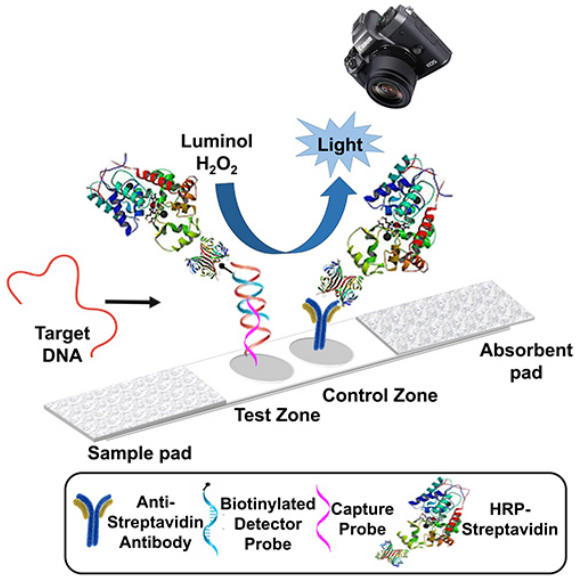


**Figure 9.** Schematic illustration of a chemiluminescence-based nucleic acid lateral flow biosensor (CL-NALFB) based on sandwich assay strategy for quantitative detection of DNA. A professional digital camera was used to acquire chemiluminescent images. The CL-NALFB can detect 10 nM to 10 pM of DNA without the target labeling.

*et al.* reported an ultrasensitive label- and PCR-free silicon nanowire optical biosensor for genome detention. This biosensor was tested with *Hepatitis B* virus whole genome and successfully exhibited a LOD of 20 copies/reaction (58).

### 4.6.1. Evaluation of cross-reactivity

To verify the specificity of the NALFB, tests were performed for the same concentrations of 5 different species including *Klebsiella pneumonia* ATCC13883, *Enterococcus faecalis* ATCC19433, *Proteus mirabilis* ATCC4371, *Serratia marcescens* ATCC13880 and *Staphylococcus haemolyticus* ATCC43252. It was found that the resulting signal in the presence of high concentrations of genomic DNA ($2\times10^5$ ng/ml) derived from these species was perfectly distinguishable as compared to the CL intensity in the presence of *E.coli* genome with the same concentration. Ultimately, the feasibility of the biosensor was also validated with another *E.coli* strain, PTCC1276. Taken together, these findings indicate the high specificity of the proposed method for *E.coli* detection (Figure 8B and C).

## 5. CONCLUSION

Our results show the great potential of the developed CL-based NALFB to detect low amounts of DNA without the need for labeling of the target DNA (Figure 9). The CL-NALFB was capable of diagnosing DNA under standard conditions with a facile procedure and total assay time of 20 to 45 minutes. Utilization of an ordinary digital camera for CL imaging, makes the developed NALFB suitable for point of care and *on-site* diagnosis. Furthermore, the enzymatic label has demonstrated to be sensitive, low-priced and suitable for quantification purposes. Over recent years, cheap and portable devices have been developed for CL quantitative analysis. It is expected that quantification of target DNA can be achieved using CL-NALFBs integrated with a portable CL detector. We hope that the proposed technique has the potential to be employed for *on-site* and rapid testing of a large variety of DNA biomarkers.

## 7. ACKNOWLEDGMENT

The authors declare that they have no Conflicting interests. We would like to appreciate Tarbiat Modares University for supporting this project.

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**Abbreviation:** CL: chemiluminescent; CL-NALFB: Chemiluminescence-based nucleic acid lateral flow biosensor; LAMP: Loop Mediated Amplification; EXPAR: Exponential Amplification Reaction; *E.coli: Escherichia coli;* PCR: Polymerase chain reaction; POCNAT: Point of care nucleic acid testing; NALFB: Nucleic acid lateral flow biosensor; SERS: Surface-Enhanced Raman Scattering; HRP: Horseradish peroxidase; CCD: Charge-coupled-device; CMOS: Complementary metal-oxide semiconductor, rRNA: *Ribosomal RNA;* ECL: Enhanced CL substrate; StAv: Streptavidin; TBS: Tris-buffered saline; LOD: Limit of detection; GNP: Gold nanoparticle; $T_h$: Hybridization temperature; $T_m$: Melting temperature; NALFIA: Fabricated a nucleic acid lateral flow immunoassay; *methicillin*-resistant *Staphylococcus aureus* (MRSA); multi signal probes (MSP)

**Key Words:** Point of care, Nucleic Acid Testing, Lateral Flow Biosensor, Chemiluminescence, 16S rRNA

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