Development of a nucleic acid-based lateral flow device as a reliable diagnostic tool for respiratory viral infections

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ABSTRACT

Viral infections continue to pose a significant threat to the public health, leading to high morbidity and mortality rates worldwide. To combat these challenges, early detection and treatment are essential in reducing hospitalizations and preventing severe complications. Simple, inexpensive, and sensitive diagnostic methods are in constant demand in many areas. In this study, we report the development of a nucleic acid-based lateral flow immunoassay device (NALFIA) and demonstrate its successful application in conjunction with a multiplexed reverse-transcription loop-mediated isothermal amplification assay (LAMP) for the detection of SARS-CoV-2 and influenza. In our approach the NALFIA part preparation is independent of the target, and has the potential to ensure widespread use in diagnostics particularly where testing speed is critical such as in respiratory viral infections.

- Simple, inexpensive, sensitive and reliable rapid diagnostic tool.
- Target independent design.
- Effective use for respiratory samples due to practical sample extraction.

Specifications table

Subject area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	Point-Of-Care Diagnostics
Name of your method:	Universal-NALFIA; U-NALF
Name and reference of original method:	Loop-Mediated Isothermal Amplification (LAMP)
	Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 2000 Jun 15;28(12):E63. doi:10.1093/nar/28.12.e63. PMID: 10871386; PMCID: PMC102748.
Resource availability:	• Colloidal gold nanoparticles, 40 nm BioReady (Nanocomposix)
	• Bst enzyme (NEB)
	• Haptens and antibodies (Vector Laboratories)
	• Labeled Primers (Sentromer DNA Technologies)
	• Airjet/Biojet XYZ 3060 Dispensing Platform (BioDot)
	• Isothermal heating box (Arnica)

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Methods details

Introduction

Early detection and treatment are critical for viral infections to reduce hospitalizations and the risk of complications. A wide range of point-of-care tests (POCTs) was developed for use as portable, handheld devices and test kits for performing diagnostic tests outside of a laboratory, some of which are not yet mature [1]. In the fight against the recent pandemic COVID-19, lateral flow (LF) tests played a very important role in isolating infected individuals to prevent the spread of the disease.

A lateral flow test is essentially an immunochromatography-based biosensor where sample may be urine, blood, saliva, sweat, serum, or other fluids. It consists of a strip of overlapping membranes that direct a drop of the sample onto the detector and capture proteins, allowing the user to see a test line and a control line, usually contained in a cassette. The test is easy to use, fast and inexpensive. Despite these advantages, there are limitations to the specificity and sensitivity of lateral flow immunoassay tests [2,3–5]. The instability of the proteins, which can be easily altered by environmental conditions such as pH, temperature, or salts that can affect ionic interactions, leads to problems with sensitivity. Then, specificity is compromised by numerous interferences with native antibodies leading to false positives and negatives. According to the World Health Organization (WHO) and the U.S. Center for Disease Control (CDC), the use of nucleic acids is the gold standard in molecular diagnostics and a better alternative to proteins in molecular biosensors because they are stable and amplifiable and therefore can be detected at very low concentrations. The level of the test line signal is directly proportional to the concentration, and it is even possible to obtain a quantitative value using an electronic sensor device [6]. Many studies reported the use of nucleic acids instead of proteins in lateral flow assays [7–17]. However, there was no significant progress or widespread application on the commercial side.

One of the reasons is the high labor intensity of assay development. There are two methods for performing nucleic acid lateral flow assays: NALF and nucleic acid lateral flow immunoassay (NALFIA). NALF contains no immunoreagents and is based solely on the hybridization kinetics of Watson-Crick base pairing. The amplicon from the PCR reaction, as a sample, is directly detected and captured by complementary oligonucleotide fragments conjugated to gold for detection and immobilized on the nitrocellulose membrane by a small molecule and its high affinity counterpart, such as biotin-streptavidin. Because hybridization occurs on paper, any formation of a secondary structure in any of the three fragments results in severe destabilization of the interactions and requires lengthy optimization for each new target. NALFIA, on the other hand, uses the molecules with established affinity for detection and capturing. Moreover, in the presence of the target nucleic acid region, the already amplified product with label is easily detected and captured by its strong acceptor binder on the nitrocellulose membrane. The architecture does not require specific optimization of the lateral flow strip, and the reagents used on the strip do not need to be target-specific.

On the user side, nucleic acid lateral flow assays, like other nucleic acid amplification-based assays (NAAT), require several steps to analyze clinical samples. The three steps 1) isolation of the target pathogen from the sample and cell wall lysis to release its nucleic acids (NA) (e.g., RNA of virus from nasal swab or DNA of bacteria from blood); 2) amplification of NAs; 3) detection of the target pathogen and control. It is imperative to simplify and reduce these steps to expand their practical use.

In this study, we report the development of a multiplex reverse transcription LAMP in conjunction with a NALFIA for the detection of SARS-CoV-2 and influenza. We also describe a universal design for NALFIA that could facilitate its broader application.

Materials and methods

The development of the nucleic acid lateral flow assay and its application are described in sequence. The application is shown in the graphical abstract.

Assay development

Reference RNA, samples and RNA release

We utilized viral RNA for SARS-CoV-2 and Influenza-A as positive control, generously provided by Prof. Damla Arısan from Gebze Technical University). To validate the extraction protocol, we used nasal swabs from confirmed COVID-19 cases via qPCR testing. These swabs were placed into 250 μl swab solution containing reagents to inactivate the virus, release the RNA and stabilize it. Before proceeding with the LAMP reaction, sample tubes were incubated in a 65 °C heater for 10 min.

Primer design and synthesis

We used LAMP Designer Software (Premier Biosoft) to design a variety of primer sets while pulling published primers from the literature to evaluate their efficiency and sensitivity. For the final study, we utilized the primers designed by Nakauchi et al. [20] for Influenza A (INFA)/M2 gene and by Rabe and Cepko [21] for SARS-CoV-2/Orf1a, enhanced by incorporating a "TTTT" linker between the F1c and F2 regions of inner primer, as demonstrated by Torres et al. (2011). As an additional modification to the original designs, we labeled the forward and reverse loop primers in both sets with Fluorescein or Digoxigenin and Biotin for NALFIA. The primer synthesis was performed by Sentromer DNA Technologies Inc. (Istanbul, Turkey) (Table 1).

Table 1

Final primer sets used in multiplex RT-LAMP reaction.

Orf1a-FIP		TCA GCA CAC AAA GCC AAA AAT TTA TTT TTC TGT GCA AAG GAA ATT AAG GAG
Orf1a-BIP		TAT TGG TGG AGC TAA ACT TAA AGC CTT TTC TGT ACA ATC CCT TTG AGT G
Orf1a-F3		CGG TGG ACA AAT TGT CAC
Orf1a-B3		CTT CTC TGG ATT TAA CAC ACT T
Orf1a-LF	FLUORESCEIN	TTA CAA GCT TAA AGA ATG TCT GAA CAC T
Orf1a-LB	BIOTIN	TTG AAT TTA GGT GAA ACA TTT GTC ACG
INFA-F3		GAC TTG AAG ATG TCT TTG C
INFA-B3		TRT TAT TTG GGT CTC CAT T
INFA-FIP		TTA GTC AGA GGT GAC ARR ATT GCA GAT CTT GAG GCT CTC
INFA-BIP		TTG TKT TCA CGC TCA CCG TGT TTG GAC AAA GCG TCT ACG
INFA-FLP	DIGOXIGENIN	GTC TTG TCT TTA GCC A
INFA-BLP	BIOTIN	CMA GTG AGC GAG GAC TG
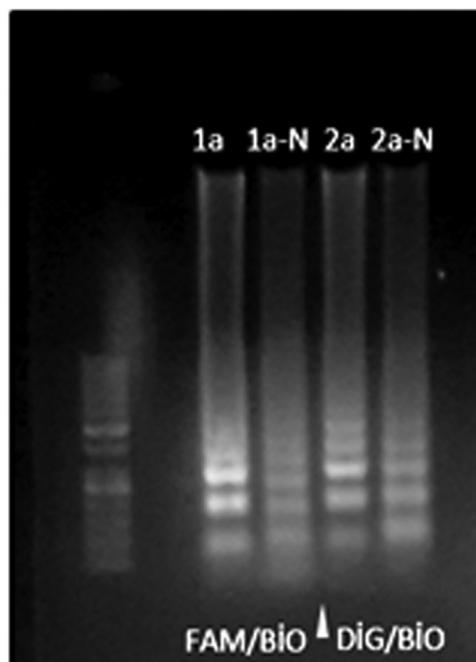
Fig. 1. LAMP reaction products on a 1% agarose gel. Amplicons with labels 1a) Fluorescein-Biotin 1a-N) NTC 2a) Digoxigenin-Biotin 2a-N) NTC.

LAMP reaction and mini heater

50 µl (2–3 drops) of the swab solution, containing the sample, was added into the LAMP reaction tube alongside the reaction reagents. Each reaction mixture was composed of 1 µL (200 U/µl) of M-MuLV Reverse Transcriptase, 1.6 µM each of FIP/BIP primers, 0.2 µM each of F3/B3 primers, and 0.4 µM of LoopF/B primers. Additionally, we included 1.4 mM dNTP Mix, 1.0 M Betaine, 6 mM MgSO4, 1 × ThermoPol buffer (20 mM Tris–HCl [pH 8.8 at 25 °C], 10 mM $(NH_4)_2SO_4$ 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100), and 8 U Bst polymerase large fragment (New England Biolabs, Ipswich, MA, USA) in the reaction mixture. A parallel reaction was set in a conventional lab heat block in a 2 mL PCR tube. Additionally, a four well mini isothermal heater was designed and a 3D printer cased working prototype was developed by Arnica Inc., manufacturer of small electronic home appliances, with our input and specifications. The reaction mixes were then incubated at 65 °C for 20 min. Subsequently, the LAMP products were run on a %1 Agarose gel for verification. (Fig. 1).

Covalent conjugation of capture protein and conjugate pad coating

To prepare the capture protein, we used 40 nm carboxyl coated gold nanospheres (BioReady Nanocomposix) and Streptavidin (SA-5000 Vector Laboratories Inc., CA, USA), as a bait for biotinylated biomolecules. Streptavidin was conjugated with colloidal AuNP using the protocol suggested by the manufacturer. First, carboxyl coated AuNP's were activated by incubating for 30 min with EDC-sulfoNHS. After centrifugation, the supernatant was removed, followed by two washes with the sodium phosphate buffer at pH 7.4. Next, the activated AuNPs were incubated with 50 µg protein for 1 h at room temperature on a rocker. After the incubation, 10 µl of 50% Hydroxylamine was added as a quencher and incubated for an additional 10 min. The particles were then centrifuged and
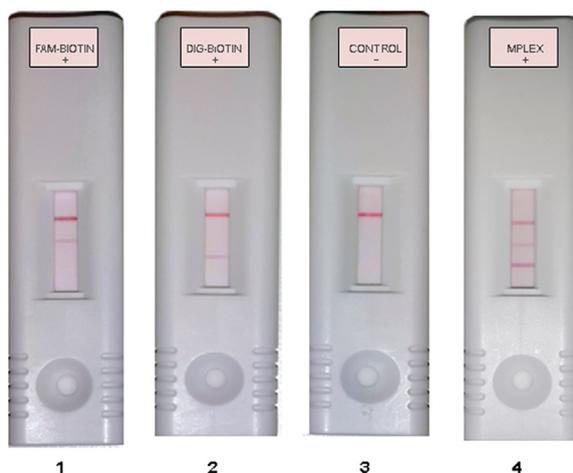
Fig. 2. NALFIA results 1) SARS-CoV-2 positive sample 2) SARS-CoV-2 negative, Influenza A positive sample 3) NTC 4) SARS-CoV-2 and Influenza A multiplex positive.
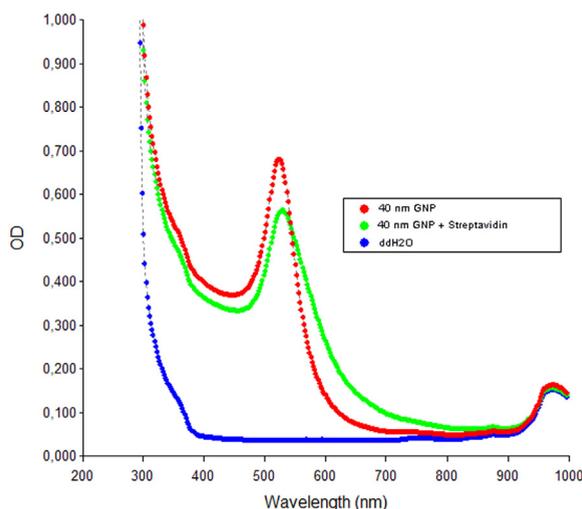
Fig. 3. UV/Vis spectrum of the conjugate.

washed twice. To prevent the nonspecific binding, a 1 h blocking step was added. After centrifugation, the pellet was resuspended using conjugate diluent buffer (0.5X PBS, 0.5% BSA, 0.5% Casein, 1% Tween 20, 0.05% azide pH 8.0). The successful conjugation was verified by measuring the absorbance at around 530 nm using Biotek Epoch spectrophotometer. A distinct red-shift in the UV–vis spectrum indicated a change in the local refractive index (Fig. 3) due to the successful conjugation. Finally, conjugate was sprayed on the conjugate pad using the BioDot AirJet XYZ 3060 Dispensing Platform (San Diego U.S.A.)

Preparation of the NALFIA test strip and cassette

Lateral flow test strips were assembled according to the optimized length of each of the membrane as sample pad, conjugate pad, nitrocellulose pad and absorbent pad (Fig. 4).

Nitrocellulose membrane streaking

The nitrocellulose membrane was streaked with capturing antibodies using a BioDot Biojet XYZ 3060 Dispensing Platform (San Diego U.S.A.) at a rate of 1 µl/cm. Each antibody as Anti-fluorescein for test line-1, Anti-digoxigenin for test line-2 and Anti-streptavidin for control line was dispensed at a concentration of 0.45 mg/ml. If instead of multiplexing only one target were to be detected then one of the test lines can be omitted. The control line takes advantage of the unbound AuNP conjugated Streptavidins.

1. Sample pad
2. Conjugate pad (GNP-Protein and MNP-Oligo)
3. Nitrocellulose membrane
4. Wick pad
5. Backing card
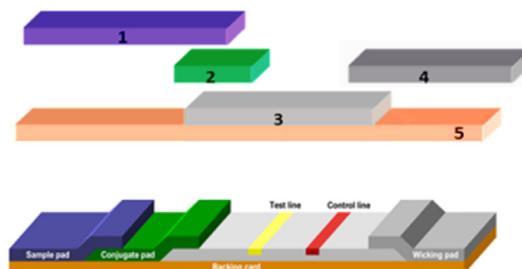
Fig. 4. Strip membrane assembly.

Method validation

Targeting gene regions instead of protein parts offers significant advantages in terms of sensitivity and specificity. Nucleic acids are well-known for their stability in various environment and contain unique regions that can be specifically identified. Several studies reported successful application of multiplexing LAMP reaction and NALFIA targeting bacteria and parasites [22,23]. The critical aspect of this study was to demonstrate a rapid diagnostic tool that ensures accurate, sensitive, and fast detection while making rapid test development as practical as possible for both diagnostic test developers and end-users, including clinicians or simply consumers and validate its use for detection viral infections.

From sample collection to signal visualization, a pipeline was established to perform reverse transcribed loop-mediated isothermal polymerase chain reaction (RT-LAMP) and nucleic acid lateral flow immunoassay (NALFIA) using a custom-designed isothermal mini-heater powered by a USB charger as visually represented in the graphical abstract.

Application

Three easy to apply steps are summarized as following;

Sample preparation

The nasopharyngeal or oropharyngealswab specimen is immersed in the specimen buffer and incubated at 65 °C for 10 min to allow the release of the nucleic acids.

Amplification reaction

After incubating the sample at 65 °C for 10 min, 2–3 drops of the sample are transferred to the reaction tube containing the LAMP reagent mixture and incubated at the same temperature, 65 °C, for 20–60 min.

Lateral flow run

The nucleic acid lateral flow assay is performed with the LAMP reaction product, which may or may not contain the amplified target nucleic acid region. 3 drops of the LAMP product are added to the sample container of the lateral flow cassette. Generally, 10 min is sufficient for the sample to flow through the membranes and the conjugate to be fully released. The reaction can have multiple sets of primers resulting in multiplex detection of targets. We have designed a simple three-line strip with one control and two test targets. With careful primer design, this method can be effectively applied to any target.

Discussion

While lateral flow tests are simple to use, constructing a lateral flow cassette de novo can be laborious involving multiple steps ranging from selecting suitable membranes for the sample type to identifying antibodies, nanoparticles, and conjugation methods. It involves many steps that need to be thoroughly mastered to achieve acceptable results. Even then tedious optimization work has to be done with systematic experimentation.

Among the clinical specimens, naso/ oro-pharyngeal swabs are the simplest to extract nucleic acids, which are commonly used in respiratory infections. However, for other types of samples such as saliva, blood, semen, and vaginal discharge, various lysis buffer formulations are available and further optimized by adjusting the surfactant and salt composition. Nowadays there are several types of polymerases that are capable of synthesis even in the presence of inhibitors, reducing the need for extra purification of genetic material. Studies confirmed that the presence of most PCR inhibitors that affect the conventional PCR reactions does not significantly affect the course of LAMP reaction [14–16]. Nasal swab sample can remain stable in the extraction tube for up to 48 h at 4–8 °C. Nucleic acids are slowly released upon short heat exposure. The qPCR studies revealed that with this swab buffer formula, 10 min at 65 °C is sufficient to detect low concentrations of virus samples.

The concept of U-NALF (U- for universal) should aid the lateral flow diagnostics to speed up the development by focusing primarily on LAMP primer design. By providing repeatable, sensitive and specific results, along with the convenience, U-NALF is expected to promote wider application of nucleic acid-based lateral flow tests.

The basic design of a lateral flow strip is shown in Fig. 3. Detection of the target molecule is ensured by a counter molecule conjugated to gold nanoparticles and sprayed on the conjugate pad . When the target molecule is present in the sample, the molecular complex conjugated to gold binds to it and is carried with the flow onto the nitrocellulose membrane, where it is captured by another specific protein.

The gold nanoparticles can be conjugated to the proteins by passive or covalent conjugation method. We employed the EDC and sulfoNHS covalent conjugation method to ensure stability. We tested different particle sizes, including 40 nm, 150 nm and 1 μm magnetic nanoparticles, all carboxyl coated, to enhance the positive signals, and found that the 40 nm gold nanoparticles performed best. Conjugates were UV/Vis measured on a Biotek Epoch Microplate Spectrophotometer (Agilent, CA. USA). Gold conjugates are expected to exhibit a minor shift compared to the bare gold. To demonstrate the utility of U-NALF, we developed a test that capable of detecting the presence of RNA from SARS-CoV-2 and Influenza-A. The design and source of LAMP primers were described in the methods section and shown in Table 1. A set of representative results using the control samples are shown on Fig. 2. We believe U-NALF method can be easily adopted for the development of nucleic acid lateral flow tests.

In a separate recent study by the authors, the performance of the SARS-CoV-2 antigen assay that was developed in the same fashion as described above was compared to real-time reverse transcriptase PCR in 200 symptomatic COVID-19 suspected patients. The rapid antigen assay showed an overall agreement rate of 100% with a sensitivity of 80,6% and a specificity of 93,7% for the samples with Ct below 30 [24]. This information is added to support the validity of the lateral flow design and materials that the authors have employed.

Conclusion

Respiratory infection-causing viruses localize in the upper respiratory tract where samples can be easily collected via nasal or oropharyngeal swabbing. Nucleic acids are easily released into the swab solution upon 5 min. incubation at 65 °C. LAMP reagents can tolerate various inhibitors that might be present in these types of sample. In this study, we successfully employed two sets of six primers (FIP, BIP, F3, B3, LOOP-F, LOOP-B) for specific and isothermal amplification of SARS-CoV-2 and Influenza-A RNA as multiplex reaction within the same tube. The amplification was performed at 65 °C for 20 min using a mini USB heating block, followed by detection on lateral flow. The practical workflow, which involves sample collection, viral lysis, nucleic acid amplification and lateral flow detection, has yielded repeatable results. These promising outcomes indicate that the NALFIA method holds the potential for broader application as a reliable point-of-care diagnostic tool [18,19].

Ethics statements

All of the experimental procedures involving pathogens and potential environmental hazards were conducted in accordance with the Ministry of Health Gen. Dir. of Public Health and related regulation and well defined procedures of the experimental laboratory operated under ISO13485 guidelines.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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