



Simple rolling circle amplification colorimetric assay based on pH for target DNA detection

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ABSTRACT

Detection and identification of DNA by PCR has opened tremendous possibilities and allows detection of minute quantities of DNA highly specifically. However, PCR remains confined to laboratory settings because of the need of thermocyclers and other analytical equipment. This led to development of isothermal amplification techniques, among which Pad Lock Probe (PLP)-based Rolling Circle Amplification (RCA) has several advantages, but typically also requires a laboratory apparatus of some sort to measure DNA amplification. To circumvent this limitation, while still taking advantage of PLP-based RCA, we developed a colorimetric assay that relies on pH change. Using this assay, we can detect DNA in the low picomolar range and obtain results observable with the naked eye in only 20 min without any requirement for a thermocycler or other complex device, making it a particularly portable assay.

1. Introduction

Because of portability, simplicity of diagnosis and efficiency in detection without requiring access to well-equipped laboratories, point of care (POC) testing is favored especially in areas with shortage of facilities [1–3]. The increasing interest in such type of diagnosis has led to develop fast and cheap devices which can analyze genetic profiles or various analytes easily and precisely. Eventually, we could relinquish the classical ways of medical diagnosis and use mostly miniaturized and label free systems [1,4]. One of the integral tools in the field of life sciences is nucleic acid testing which is mainly based on PCR and is highly applicable either in research or clinical purposes. However, PCR needs sophisticated and expensive thermal cycler machines and centralized laboratories with professional personnel. Therefore, application of POC nucleic acid tests which use isothermal DNA amplification methods are on rise due to their simplicity and the fact that they do not require any special devices [5,6]. These alternative techniques have been widely used for monitoring of diseases and mainly infectious diseases in developing countries [1,5].

In the 1990's, an exclusive enzyme was introduced which can make tandem repeat single strand DNA (ssDNA) from circular DNA templates. This isothermal DNA amplification process which is called rolling circle amplification (RCA) is based on a circular DNA template and utilizes specific DNA polymerases (Bst, Vent exo- and Phi29 DNA polymerases) to elongate DNA strands either in linear (LRCA) mode or

hyperbranched (HRCA) mode [7,8]. Among these two models, HRCA is more efficient and powerful in signal magnification and generates more products from each circular template [8,9]. In view of the fact that RCA is based on the circular DNA template, in 1994 a long circularizable probe was proposed which is called padlock probe [10]. This circularizable probe forms circular DNA after specific recognition of target which then is utilized as a template for signal amplification via RCA [11,12].

Like these two previous studies, in this work H₅N₁ influenza virus was targeted because this virus is still a major concern among scientists and public [13,14]. However, due to high pathogenicity and virulence of H₅N₁ virus [15], in this study synthetic H₅N₁ target was employed for optimization of the colorimetric assay and the M13 bacteriophage genome was used as a model for evaluating the performance of the proposed method in real-sample condition.

Broad applications exist for pH sensitive dyes in different fields including determining pH of solutions, freshwater, pools, determination of CO₂ and SO₂ in air, developing pH-based sensors which usually work and respond based on alterations in the hydrogen ions or hydroxyl ions of the environment [16–19]. On the other hand, other sensors are developed for different ion detection such as magnesium or cadmium [20,21].

In 2015, Tanner et al. introduced a new method for visual monitoring of loop mediated isothermal amplification (LAMP) through inherent production of hydrogen ions by DNA polymerase during DNA

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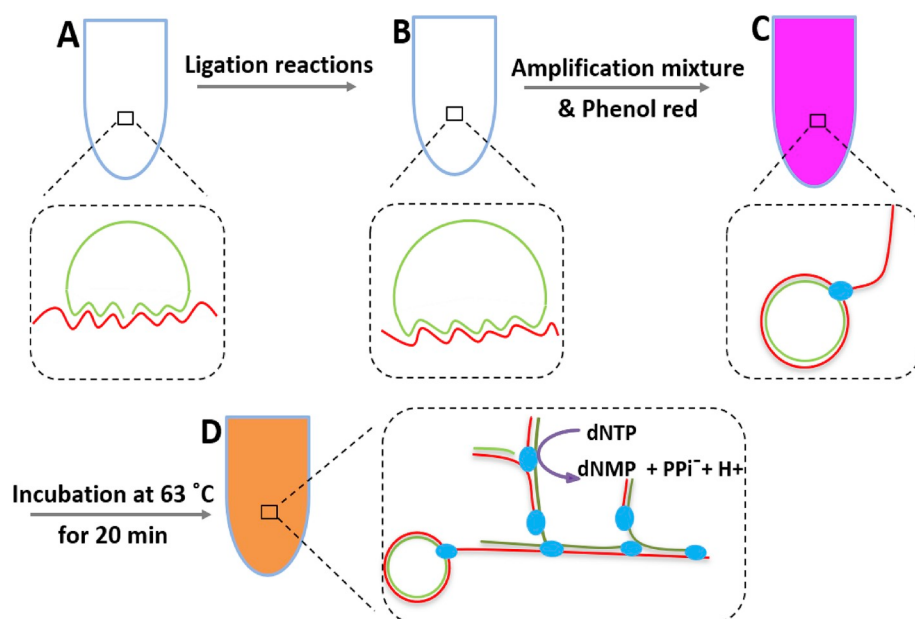


Fig. 1. Representation of colorimetric assay with phenol red. (A) In the first step the hybridization reaction is carried out. In this situation PLP (green line) and H_5N_1 target (red line) can be gradually and specifically hybridized. (B) After hybridization the mixture is treated to ligate the two ends of the PLP and generate a circular template for RCA reaction. (C and D) Afterward, amplification components including Phenol red are introduced to the ligation mixture. The Bst 2.0 enzyme (blue ellipse) initiates RCA by utilizing circular PLP as a template. During amplification, Bst 2.0 uses dNTPs to incorporate dNMPs into DNA chains and releases PP_i^- and H^+ as by-products, thus decreasing pH. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

amplification and pH indicator dyes (phenol red, cresol red, neutral red or m-cresol purple) [22]. Combination of pH sensitive dyes with LAMP, multiple displacement amplification (MDA), strand-displacement amplification (SDA) and PCR have been applied for developing colorimetric biosensors [6,22–28], but in the current work this strategy is exploited for the first time with RCA based on Pad Lock Probes (PLPs) (Fig. 1). Also, in this project for the first-time DNA ligation has been done in a reaction mixture lacking Tris-HCl (or other typical buffering agent) to be able to develop a colorimetric assay using phenol red with PLP-based RCA. Furthermore, by using this strategy a new colorimetric method was introduced for specific monitoring of SNPs, highlighting the advantage of PLP colorimetric RCA assays over other isothermal amplification methods. Finally, we have shown the robustness of the proposed assay with different types of samples.

2. Material and methods

2.1. Probes and primers

The phosphorylated H_5N_1 PLP has specific arms complementary to the synthetic H_5N_1 target primers, which corresponds to a sequence in the hemagglutinin (HA) gene from H_5N_1 genome [13,14,29,30]. Also, M13 PLP and synthetic M13 targets were selected from a conserved part of the M13 bacteriophage genome and this recognition sequence was also verified by PCR in the presence of real samples. All oligonucleotides including PLPs, forward and reverse primers and synthetic targets were provided from Sigma Aldrich Company and Integrated DNA Technologies (IDT) (Table S1).

2.2. M13 bacteriophage genome isolation

M13 bacteriophage solution was first treated with 50 μ g/mL proteinase K per reaction (Bio Basic, Canada) at 37 °C for 60 min and then the genome was extracted using phenol: chloroform extraction (phenol, Bio Basic, Canada; chloroform: Sigma-Aldrich, USA) [31] and then the extracted DNA was precipitated by utilizing conventional precipitation with ethanol [32]. Thereafter, the concentration of the extracted genome was determined using a Nanodrop device (Thermo Fisher Scientific, USA).

2.3. Ligation and amplification reactions

In previous studies pH shock was used instead of heat shock for simplification of the whole detection procedure [13,14]. However, in the current work we employed heat shock for DNA denaturation since the signal is highly dependent on the pH change during the amplification reaction. For the ligation, 1 μ L of target DNA, 2 μ L of deionized water and 1 μ L PLP (at final concentration of 0.075 μ M) were added to the 5 μ L of 2X “no-Tris” ligation solution (20 mM $MgCl_2$ (Fisher Scientific, USA), 2 mM ATP, 20 mM DTT (Bio Basic, Canada), pH 8.50). Thereafter, reaction mixtures were incubated at 95 °C for 5 min then gradually cooled down to room temperature to allow hybridization between PLP and the target. This was followed by addition of 1 μ L (5 units) of T4 DNA ligase (New England Biolabs, USA) and placing the reaction for 60 min at room temperature [33]. In this assay exonuclease reaction using Exonuclease I enzyme was not used due to two reasons: i) minimizing introduction of Tris-HCl to the reaction mixture which is already added to the enzyme stock by the company; and ii) the Bst DNA polymerase that is used in this assay can amplify circularized PLP even in a topologically constrained situation and thus there is no need for exonuclease enzymes prior to the amplification reaction [34]. This phenomenon was confirmed by using an extended synthetic M13 target to determine a M13 standard curve. Afterward, 30 μ L of 2X amplification reaction solution (2.8 mM deoxynucleotide triphosphate (dNTP), 20 mM $(NH_4)_2SO_4$, 16 mM $MgSO_4$, 100 mM KCl, 0.2% v/v Tween 20 (Fisher Scientific, USA), pH 8.2) as well as 1.6 μ M forward and reverse primers, 0.1 μ M phenol red and 8 units of Bst 2.0 WarmStart (WS) DNA polymerase enzyme (New England Biolabs, USA) were added to the ligation mixture in a final volume of 40 μ L [6,22]. The amplification reaction is carried out at 63 °C for 1 h by utilizing a thermal cycler (C1000 Touch, Bio-Rad, USA) to adjust temperature and then reaction mixtures are poured into the 384 well plates (Greiner Bio-One, Germany) in order to determine the absorbance intensity between 500 and 600 nm using a plate reader device (Infinite M 1000 PRO, TECAN, Switzerland).

2.4. Visualization with gel electrophoresis

Evaluation of the performance of different ligation solutions was performed in the presence of 0.1 μ M H_5N_1 PLP and target. The gel electrophoresis was done in 2% agarose gel with 1X TAE buffer (40 mM

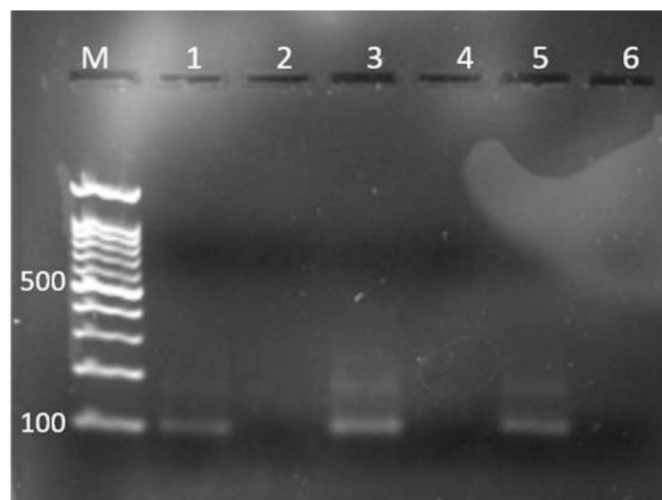


Fig. 2. Ligation reactions. Lane M: Marker, Lanes 1, 3 and 5: positive samples (with H_5N_1 target), Lanes 2, 4 and 6: Negative samples (without H_5N_1 target). Lanes 1 and 2: ligation reaction in 2X ligation solution with no-Tris (pH 8.5); lanes 3 and 4: ligation reaction with 2X ligation solution with NaCl/no-Tris (pH 8.5); and lanes 5 and 6: 10X buffer provided by the company. All ligation samples were done in 1X of the indicated buffer and treated with Exonuclease I enzyme before loading in gel. The electrophoresis has been done in 2% agarose gel for 45 min at a voltage of 120 V.

Tris-acetate, 1 mM EDTA, pH 8.0, Fisher Scientific, USA) for 45 min at 120 V.

3. Results and discussion

3.1. Optimization of ligation reaction

In contrast with LAMP reaction which is only composed of an amplification reaction, in the PLP-based RCA method it is necessary to perform a ligation reaction prior to the amplification reaction [35,36]. Therefore, it is critical to adapt both reactions with this colorimetric technique to be able to witness colour changes in phenol red resulting from the amplification reaction. For this, we optimized the ligation reaction and were able to ligate DNA in absence of Tris-HCl (normally used to buffer pH of T4 DNA ligase at 7.5) to prevent buffering of pH during amplification. In order to optimize PLP ligation-based RCA for our colorimetric assay with phenol red, the ligation reaction was carried out with the H_5N_1 target in three different situations (Fig. 2). Ligation reactions were performed with 2X no-Tris ligation solution (Lanes 1 and 2), with 2X NaCl/no-Tris (includes 100 mM NaCl) to improve ionic capacity of the ligation solution (Lanes 3 and 4) and with 10X T4 ligase buffer supplied by NEB (Lanes 5 and 6). Each reaction was also performed in the absence of H_5N_1 target as negative controls (Lanes 2, 4, 6) and each sample was treated with 10 units of Exonuclease I enzyme [37] (New England Biolabs, USA) to degrade un-ligated PLPs.

To achieve PLP ligation after omitting Tris-HCl from T4 ligase reaction mixture, two strategies were utilized. Firstly, using a 2X ligation solution instead of 10X. This strategy improves control over possible pH change after addition of the rest of ligation mixtures. Because in this situation half of the reaction is encompassed of ligation solution, the reaction is less prone to sudden pH changes due to introduction of other elements. Secondly, adjusting pH of the ligation solution at the initial pH of 8.5 instead of 7.5. Using a pH of 8.5, rather than 8.0 or 8.1 (which would be just above the phenol red upper range) also could help the ligation solution have more room for possible alterations of pH since the reaction mixture is merely composed of $MgCl_2$, DTT and ATP. Absence of dedicated buffering agents makes this new ligation solution

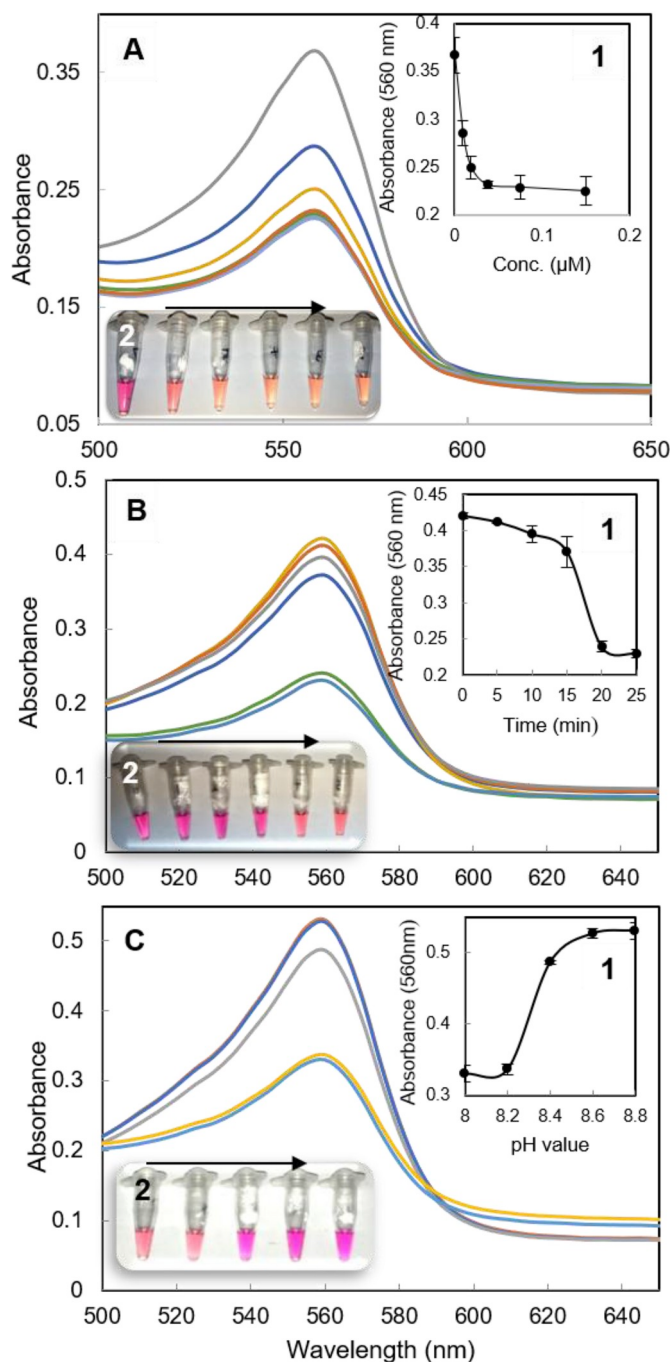


Fig. 3. Optimization of analytical parameters for colorimetric assay. (A) Optimization of PLP concentration was performed at a constant concentration of H_5N_1 target (0.25 μ M) and different amounts of PLP (from top to bottom: 0, 0.009, 0.018, 0.037, 0.075, 0.150 μ M). (B) HRCA amplification time optimization was carried out at the optimized concentration of 0.075 μ M of PLP and 0.25 μ M of target and different amplification times of 0, 5, 10, 15, 20, 25 min (from top to bottom). (C) pH of amplification buffer optimization was adjusted at a constant concentration of target (0.25 μ M) and optimized concentration of PLP (75 nM), amplification time of 20 min and in the presence of different pH of amplification reaction 8.8, 8.6, 8.4, 8.2 to 8.0 (from top to bottom). Insets 1 and 2 in graphs A, B, C illustrate the absorbance of samples at 560 nm and a picture of the tubes, respectively (arrows indicate the direction of increasing PLP concentration, amplification time and pH of amplification buffer).

sensitive to pH variations and these approaches are important to aid T4 DNA ligase reactions and provide a ligation efficiency identical to the commercial buffer.

3.2. Colorimetric tracking of RCA with phenol red

Phenol red is a well-known pH indicator with a pH shift range from 6.4 to 8.0. This pH sensitive dye is red (absorbance peak at $\lambda_1 = 560$ nm) at the upper limit of the pH range and yellow (absorbance peak at $\lambda_2 = 440$ nm) at the lower limit of its pH transition range [23]. In this project, λ_1 has been selected as the optimum extinction coefficient to track amplification reaction because absorbance intensity for λ_1 is much higher than that of λ_2 [16] and also displays sharper differences to the pH change from 5 to 8 [17]. Therefore, this wavelength is a better indicator for colour change of phenol red during RCA reaction and it is more associated to the concentration of the target.

In order to use phenol red for colorimetric detection of RCA, the pH of the amplification reaction should be adjusted slightly above pH 8.0, which coincides with the optimum pH 8.2 found for our colorimetric assay (Fig. 3C). Furthermore, a pH 8.2 is compatible with the optimum pH condition for the activity of the Bst 2.0 Warm Start (WS) DNA polymerase enzyme which is 8.8 [13,22] and at this pH the dye has pink colour due to the fact that it is above the phenol red pH transition range [23,24]. Thus, at the beginning of the amplification reaction samples are pink, as illustrated in Fig. 1C and observable in negative controls from insets. However, during the amplification reaction and incorporation of deoxynucleotides to the strands of DNA, pyrophosphates and protons (hydrogen ions) are produced as by-products [6,13,23,24], as a result the pH of positive samples with specific targets decreases overtime due to generation of protons via exponential RCA reaction. Indeed, the production of hydrogen ions is high enough to change the pH from ~ 8.2 to ~ 6.5 and consequently the colour of solution from pink to light orange which is easy to discern by the naked eye (Fig. 1D and positive samples in insets) [22,23]. This phenomenon causes a decrease of absorbance intensity at 560 nm [38] and leads to reduced signal intensity as the amplification reaction progresses, which was also confirmed by phenol red titration with pyrophosphoric acid concentration similar to that produced by DNA amplification (data not shown).

3.3. Optimization of colorimetric assay amplification

One of the important parameters in PLP-based RCA assays that has a pervasive effect in sensitivity is PLP concentration [13,14]. Selectivity of RCA is grounded on specific and precise hybridization of PLP with its target which highlights the importance of this factor optimization [37]. We optimized concentration of PLP for a constant concentration of H_5N_1 target (0.25 μ M) and different concentrations of PLP from 9 nM to 150 nM. Incrementation of the concentration of PLP is accompanied by a declining intensity at 560 nm (our detected signal) and it reaches a plateau at 37 nM (Fig. 3A). Because 37 nM is located at the lower limit of the plateau and may be more subject to disparities due to various small laboratory variations, the next point (75 nM) was deemed more robust as the optimized PLP concentration for the remaining optimization and calibration curves (Fig. 3A, Inset 1). This trend is also observable via colour change of samples (Fig. 3A, Inset 2).

As mentioned before, the signal in this colorimetric assay is in proportion with production of protons during isothermal DNA amplification reaction through incorporation of dNTPs into DNA strands [6,24]. So, we evaluated the HRCA amplification time required to generate enough protons to witness colour change from pink to light orange. Absorbance intensity gradually decreases from 0 to 15 min, whereas at the time point of 20 min, the intensity suddenly drops (Fig. 3B, Inset 1) resulting in a colour change of samples which is clearly apparent by naked eyes (Fig. 3B, Inset 2). In this experiment the concentration of target was high enough to be discriminated after 20 min, whereas for lower range concentrations more time would be needed to see the colour change [22]. On the other hand, it is reported that incubating samples for more than 60 min at 63 °C could result in non-specific amplifications as the result of primers and conditions of

reaction [6,39]. This non-specific amplification could affect colour change in negative controls in a long period of incubation [6].

The last and main factor that was optimized in this project was initial pH of amplification mixture. Investigating this parameter is not only critical due to a pH-based detection but is also particularly challenging because of the two different reaction mixtures required, namely ligation and amplification. It is essential to have an initial pH value above the transition pH range of phenol red to have pink colour at the beginning of the reaction and light orange colour after HRCA reaction. It is also necessary for the optimized pH to be close to the pH range of Bst 2.0 WS DNA polymerase activity to make it possible for the polymerase enzyme to perform HRCA [22–24]. As a result, this parameter has dual impacts in the efficiency of phenol red-based RCA detection. The HRCA reaction does not affect absorbance intensity for initial pH values of 8.8 and 8.6 and it decreases a bit for pH of 8.4. Conversely, the intensity drops significantly for an initial pH value of 8.2 and remains at the same level for pH 8.0, where the intensity reaches a plateau. We have thus chosen 8.2 as an optimal initial pH for the amplification mixture (Fig. 3C, Inset 1). The primary pH of 8.8, 8.6 and 8.4 appeared too high to witness a colour change even after amplification reaction when exponential amounts of protons are produced. On the other hand, pH of 8.2 and 8.0 were low enough to permit a pH change in the transition range of phenol red and that is why they underwent colour change from pink to light orange after HRCA reaction (Fig. 3C, Inset 2).

3.4. Calibration curve in phenol red-based RCA assay

After optimization of several parameters for the colorimetric assay, a standard curve was done with H_5N_1 as a target from 0.15 nM (and 0) to 625 nM to determine limit of detection (LOD), sensitivity and linear range of the proposed colorimetric assay (Fig. 4, Inset A). Phenol red absorbance intensity at 560 nm is concomitant with the target concentration and decreases by increasing the concentration of target. The logarithmic calibration curve highlights good linearity from 0.61 to 78.1 nM (Fig. 4B). The LOD of the system was also calculated by using

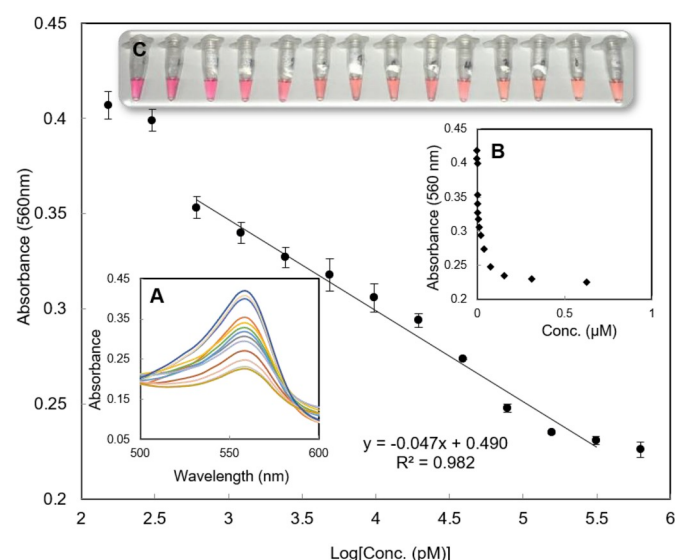


Fig. 4. Calibration curve of H_5N_1 . (A) Determination of absorbance intensity for phenol red at different concentration of targets, from top to bottom, 0, 0.15, 0.30, 0.61, 1.22, 2.44, 4.88, 9.76, 19.53, 39.06, 78.12, 156.25, 312.5 and 625 nM. (B) Standard curve for determination of H_5N_1 target in colorimetric assay. (C) Changes in colors of samples by increasing the concentration of target (from left to right). The main graph represents the data in B on a logarithmic scale. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

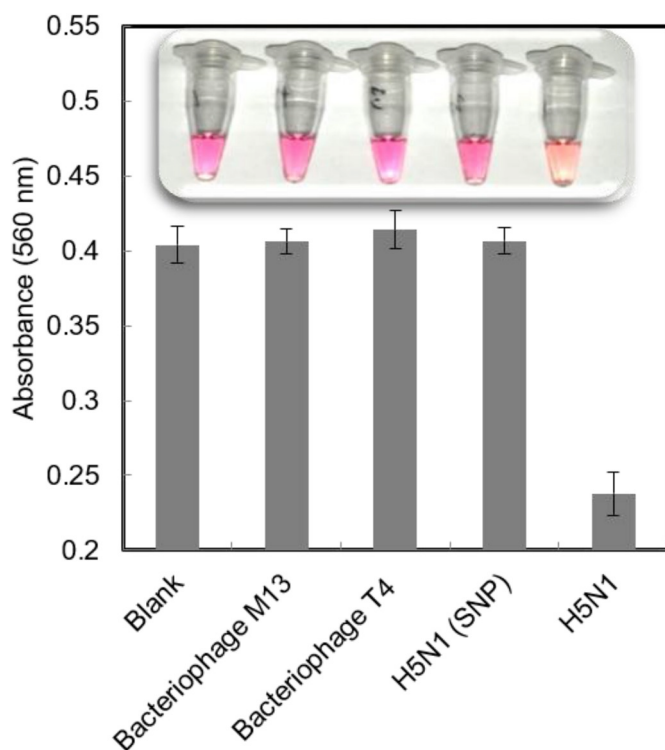


Fig. 5. Selectivity of the assay in the presence of different DNA samples. Selectivity of the system has been evaluated in the presence of different DNA molecules for the PLP targeting H₅N₁. These DNA molecules are the bacteriophage M13 and T4 genome, as well as a mutated H₅N₁ target with a SNP at the ligation site, blank control had no DNA except for PLP and HRCA oligonucleotides. Image shows changes in the colour of the sample with specific target, however the colour for the un-specific targets remains unchanged (tubes are in the same order as the histogram). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

LOD = 3 s/m (where “s” is standard deviation and “m” is the slope of the linear range) [40]. Using this equation, we estimate a LOD of 3.3 pM at the signal to noise of three. However, for such concentrations to be properly determined, further optimization of the assay would be required because it is currently within three times standard deviation of background noise according to empirical measurements.

The trend of post-HRCA colour gradually changes from pink to light orange by increasing the concentration of target. Importantly, the positive sample can be detected at 0.61 nM via an unaided eye where the absorbance intensity abruptly drops (Fig. 4, Inset C).

3.5. Selectivity of the colorimetric biosensor

Selectivity is regarded as one of the significant characteristics of biosensors which means that the biosensors only respond in the presence of specific targets. Selectivity of this colorimetric assay was verified with various DNA sequences (unrelated viruses, as well as H₅N₁ target with a SNP at the ligation site). The absorbance intensity drops only for the sample containing the exact H₅N₁ target, while for the rest of the non-specific targets the intensity is the same as for the negative control (Fig. 5). This can also be seen by an unaided eye (Fig. 5, Inset), showing the use of this simple assay that requires no complex devices to evaluate the presence of a given target sequence. The fact that the colour change has not been observed in the sample that contains a single point mutation further supports that the assay can even be used for SNP detection.

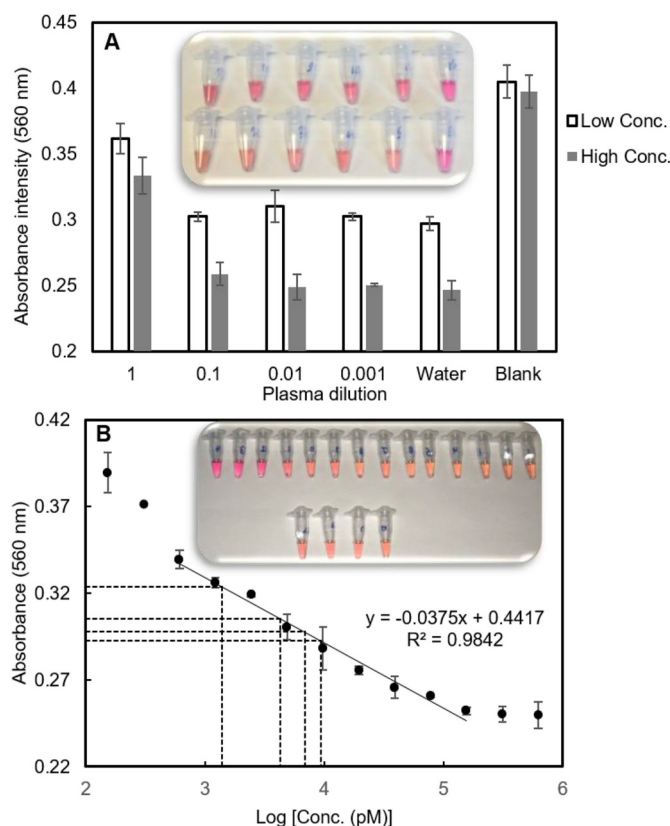


Fig. 6. Evaluating realistic samples. (A) The response was evaluated by adding 1 μ L of different dilutions of plasma including direct plasma (1), 1/10, 1/100 and 1/1000 as well as a sample without plasma as a positive control and negative control without plasma nor H₅N₁ target. This experiment has been done at 156 nM (high concentration) and 2.4 nM (low concentration) of H₅N₁ target. Inset depicts the tubes with high concentration of target (upper samples) and low concentration of target (lower samples) in this experiment, in the same order as the bar graph. (B) Standard plot for M13 phage. Standard curve for M13 phage was realized by determining phenol red absorbance of samples at different concentrations of M13 synthetic targets, from left to right, 0, 0.15, 0.30, 0.61, 1.22, 2.44, 4.88, 9.76, 19.53, 39.06, 78.12 and 625 nM (black dots). Also, four samples which contain the full genome of M13 bacteriophage were used to determine the efficiency of the proposed biosensor in the presence of real M13 full circular genome target (dashed lines). Inset shows changing of colors of tubes from red to light orange by increasing the concentration of target (upper tubes correspond to synthetic target and lower ones to real M13 full genome). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.6. Colorimetric assay with real sample

The performance of the proposed colorimetric assay in real samples was evaluated in two ways to see how it works in a matrix. First, we evaluated two different concentrations of H₅N₁ synthetic target (156 nM and 2.4 nM) in different dilutions of rat plasma. This evaluation was done due to the fact that the response of this assay is based on the change of pH and as a result including plasma, which is naturally buffered, could increase the buffering capacity of the reaction and consequently reduce intensity of the signal. As depicted in Fig. 6A, the signal intensity for the dilutions of 1/10, 1/100 and 1/1000 are almost the same as the signal intensity for the positive control signal that lacks plasma. However, the OD for the samples where undiluted plasma was added directly to the reaction is higher. This is likely due to the intrinsic coloration and heterogenous composition of plasma, making it a bit cloudy which could have an impact on the absorbance of the samples. Nevertheless, the colour change for the undiluted plasma sample is still

Table 1

Comparison of different parameters of the current work with those that have been reported in the literature.

Type of target	Amplification method	Detection method	Type of assay	Colour change	Linear range	LOD (pM)	Reference
				Portability			
DNA	HRCA	Colorimetric (HNB) ^a	Qualitative & quantitative	Violet to sky blue +	0.16–1.20 (pM)	0.028	[13]
DNA	SDA ^b	Colorimetric (ATBS ²⁻) ^c	Qualitative & quantitative	Colorless to green +	1–100 (nM)	4	[41]
Protein	HRCA	Fluorescence (SYBR Green)	Quantitative	–	0.4–80 (nM)	400	[42]
Protein	LRCA	Electrochemical (Graphene)	Quantitative	–	10–200 (pM)	10	[43]
Protein	SDA	Colorimetric (AuNP) ^d	Qualitative & quantitative	Red to purple +	2.0–80 (nM)	1100	[44]
DNA	HCR ^e	Colorimetric (AuNP)	Qualitative & quantitative	Red to purple +	–	500	[45]
DNA	(Exo III)-aided Amplification ^f	Chemilumi-nescence (Luminol-H ₂ O ₂)	Quantitative	–	0.01–1.0 (pM)	0.08	[46]
DNA	LAMP	Colorimetric (Calcein)	Qualitative	Dark yellow to yellow +	–	0.85	[47]
DNA	HRCA	Colorimetric (Phenol red)	Qualitative & quantitative	Pink to light orange +	0.61–78.12 (nM)	3.3	Current work

^a Hydroxy naphthol blue.^b Strand displacement amplification.^c 3-ethylbenzothiazoline-6-sulfonic acid.^d Gold nanoparticle.^e Hybridization chain reaction.^f Exonuclease III-Assisted Cascade Signal Amplification.

visible to the naked eye and it is comparable with the other samples that have lower dilutions of plasma (Fig. 6A, Inset). This result shows that this pH based colorimetric biosensor can work in complex situations which is the ultimate application for the proposed assay. We also tested our assay with another virus sequence for which full viruses were readily available, the M13 bacteriophage. Purified M13 phage genome was used as a model sample to simulate the performance of the assay in the presence of a real full length target. Therefore, another PLP was designed for M13 genome and response of the system was investigated in presence of different dilutions of samples of M13 phage in which the concentration was determined by a Nanodrop device. To compare results from the M13 genome, another standard curve was realized with oligonucleotides with the corresponding M13 sequence (Fig. 6B). Synthetic M13 target with extended ends were utilized in this assay to have DNA overhangs as would be the case with the M13 genome. Based on the calibration plot the concentration for four M13 real samples were 1.38 ± 0.09 , 4.29 ± 0.3 , 6.9 ± 1.3 and 9.3 ± 0.7 nM in which the concentrations were determined to be 2.50, 3.75, 5.00, 6.25 nM by a Nanodrop device. Therefore, a generally good consistency was obtained for assessing M13 genome concentration ($+/- \sim 30\%$ compared to short oligonucleotide, with good linearity as well over the range of three folds tested) which confirms the potential of this assay in different complex situations. Note that the excellent correspondence of the M13 and H₅N₁ standard curves suggests that the detection (whether quantitative or qualitative) is independent of the target and PLP sequences.

4. Conclusion

As compared with previous work shown in Table 1 [13,41–47], we improve many analytical parameters, depending on the assay comparisons. In particular, the linear range, and enhanced contrast between positive and negative samples have improved. Reported uses of other dyes namely hydroxy naphthol blue (HNB), malachite green and intercalating dyes [6] have not achieved this level of contrast. In addition, during this isothermal DNA amplification, signal is magnified enough to monitor trace amounts of the target whether in qualitative or in quantitative mode. As a result, this colorimetric assay offers a portable diagnostic technique that can be done in remote areas and with elementary

instruments. In comparison with the large fragment of DNA polymerase (wild-type) and Bst 2.0, Bst 2.0 Warm Start DNA polymerase, the latest version of this enzyme, has been reported to be best because of a higher and faster amplification rate and the ability to preserve its amplification rate even after 2 h of pre-incubation at room temperature [48]. That is why the colour of positive samples turns into light orange after only 20 min of incubation at 63 °C. Using a highly concentrated Bst 2.0 WS enzyme (120,000 U/ml) and T4 DNA ligase enzyme (400,000 U/ml) lessened additional introduction of Tris-HCl into reaction which has pervasive effect on the buffer capacity of solutions and consequently on the pH-based signal [22]. Moreover, in this work ligation reaction has been done efficiently in a reaction mixture without Tris-HCl or other buffering agents, enabling us to adopt RCA with the current colorimetric detection method using pH sensitive dyes. Although a previous report claimed that colorimetric detection using pH sensitive dyes applies to all isothermal amplification method including RCA [23], this previous work was just done on LAMP technique and does not explain how to adapt the ligation reaction with the amplification reaction, which is one of the most critical parts of the current work. So far, the PLP-based RCA method has been widely used for specific detection of SNPs in genomic DNA with high sensitivity [8,49,50]. In this work a new colorimetric method is established for the first time, with the possibility of monitoring SNPs, via a phenol red based RCA strategy that in turn further simplifies analysis of genetic material in a portable mode.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2019.04.003>.

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