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Real-time detection of H₅N₁ influenza virus through hyperbranched rolling circle amplification

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Abstract

An isothermal amplification method was developed for sensitive detection of H₅N₁ influenza virus. The padlock probe specifically bound to H₅N₁ target and circularized with T₄ DNA ligase enzyme. Then this circular probe was amplified by hyper-branched rolling circle amplification (HRCA) using Phi29 DNA polymerase. Fluorescence intensity was recorded in different intervals by intercalation of SYBR green molecules in double stranded product of HRCA reaction. At optimum time of 88 min a calibration plot with fine linearity was obtained. Using HRCA based on padlock probe and Phi29 DNA polymerase a high selectivity and sensitivity was obtained. The biosensor response was linear toward H₅N₁ in the concentration range from 10 fM to 0.25 pM with a detection limit of 9 fM at a signal/noise ratio of 3. By replacing the heat shock with pH shock, not only the procedure for detection of H₅N₁ influenza was simplified but also it could protect the DNA molecules from possible breaking at high temperature.

Keywords: H₅N₁; Padlock probe; Hyperbranched rolling circle amplification; Phi29 DNA polymerase; pH shock

Introduction

In the early 21st century, propagation of avian influenza virus (AIV) involved more than 200 million birds and then, 50 million birds died in European Union within 4 years.^{1,2} Influenza virus belongs to the orthomyxoviridae family and is classified base on its genome structure in types A, B and C.^{1,3,4} Type A is also classified base on the two different surface antigens hemagglutinin (HA) and neuraminidase (NA). Today, there are 16 known subtypes HA (H1–H16) and 9 subtypes known NA (N1–N9).⁵⁻⁷ Among them influenza A virus, subtype H₅N₁, also known as bird flu or A(H₅N₁), is a highly contagious virus found in wild aquatic birds, domestic poultry, and other animal species. The genome of this virus remains stable in these birds.^{1,8} Although transition of H₅N₁ to human occurred rarely, but in the event it causes great fear and panic due to the high fatality. For instance, it caused the death of 6 out of 18 people infected in Hong Kong in 1997.^{1,9-11}

Diagnostic methods based on DNA amplification have widely changed scientists view in viral diagnosis.¹²⁻¹⁹ In contrast to polymerase chain reaction (PCR) based DNA diagnostics which needs special instrumentation to cycle the temperature, RCA is a powerful and simple technique in which amplification is occurred in isothermal condition.^{15,20-23} The amplification process in RCA could be performed either by linear RCA (LRCA) or hyperbranched RCA (HRCA). In LRCA, one primer initiates the reaction and produce long single strand DNA; but, in HRCA more than one primer start the reaction and produce high-molecular-weight double-stranded DNA that contains multiple copies of circular DNA.²⁴⁻²⁹ By using HRCA it is possible to generate more than 10⁹ copies of each circle in 90 minutes which results a complex pattern of DNA strand displacement.²⁵ It is also possible to follow the RCA in real-time, and quantify the

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3 amplification for determination of the target concentration. For this, the synthesized fluorogenic
4 indicators such as molecular beacons or SYBR green could be utilized.³⁰⁻³⁶
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9 Padlock probe (PLP) is a single strand circular DNA template which was first introduced by
10 Nilsson et al. in 1994. It consists of two specific arms that placed at 3' and 5' sites and are
11 connected to each other via a target-non-complementary sequence then, by action of DNA ligase
12 the PLP is formed in a circular shape. This process led to recognize the target molecules very
13 efficiently.^{24,37-39}
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21 In the present study, by using PLP, HRCA and Phi29 DNA polymerase a simple RCA
22 technique was developed for real-time detection of H₅N₁ at femto level. Generally, for DNA
23 denaturation heat shock is used. However, this technique possibly causes breaking in DNA
24 molecules due to applying high temperature. This probably decreases the efficiency of RCA
25 reaction. In the present work to overcome this defect, the pH shock process was replaced with
26 heat shock for DNA denaturation. In addition, in most published results^{14,27-29,35,40-43} BstI DNA
27 polymerase, with the optimum temperature of 63-65 °C, was applied for HRCA. But in the
28 present work this enzyme was replaced with Phi29 DNA polymerase works at room temperature
29 however its optimum temperature is 30-37 °C. Furthermore, unlike BstI DNA polymerase the
30 recent enzyme does not need exonuclease treatment before HRCA reaction and also has high
31 ability in amplification of DNA.
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48 **Results and Discussion**

49 *PLP circularization and HRCA amplification*

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52 In order to monitor PLP circularization, the ligation products were separated and monitored
53 using agarose gel. In Fig. 1A, Lane 1, PLP was loaded in the linear form. This was shown as **a** in
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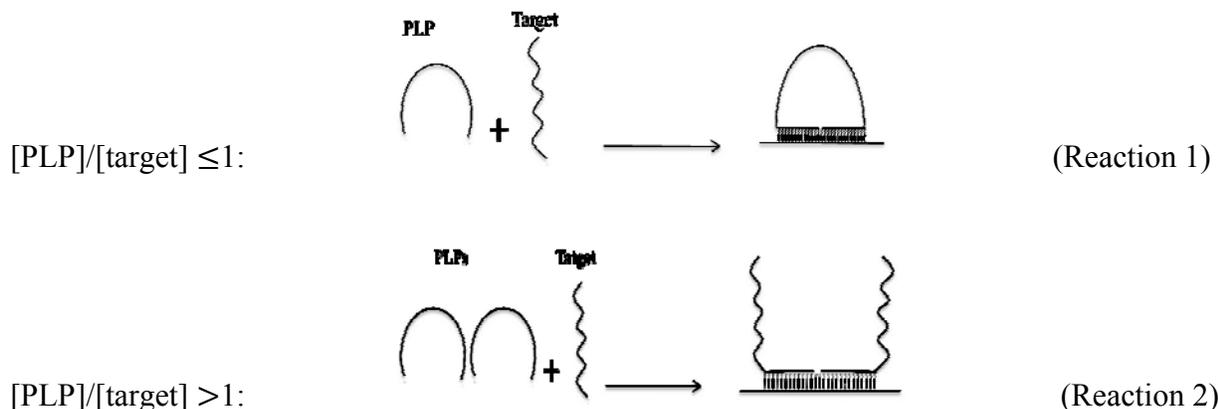
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3 Fig. 1. Then, at the end of electrophoresis its position was used as a control to detect the linear
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5 PLP (L-PLP) position in the backside of C-PLP. It was observed that C-PLP moved faster than
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7 L-PLP because of the packed nature of C-PLP and due to the circularizing process.^{24,30}
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10 Consequently, at the end of electrophoresis, the C-PLP (Fig. 1a, lane 2) was more precursor than
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12 L-PLP (Fig. 1a, lane 1).
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16 To monitor the isothermal amplification of HRCA reaction, two similar HRCA samples
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18 were prepared within 100 min and then HRCA products with a wide spectrum of molecular
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20 weight have been loaded into the gel. As seen in Fig. 1 (step V), RCA products are produced
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22 in concatemeric double strand DNA form. Therefore, these products have such a high molecular
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24 weight, so that some of the RCA products have remained in the gel and some of them which are
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26 in lower molecular weight made smear in backside of the main product (Fig. 1b, lane 2, 3).
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31 *Optimization of PLP concentration*

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34 In order to assay H₅N₁ the effective parameters for quantification of synthetic target were
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36 optimized. At first, the PLP concentration change was studied by recording the fluorescence
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38 intensity against time. The HRCA reaction was carried out at 37 °C in the presence of constant
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40 concentration of target but different concentrations of PLP (Fig. 2). As seen in the Inset of Fig. 2,
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42 the extra amount of PLP leads to decrease the fluorescent signal. To understand the reason for
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44 such a decrease in signal, one should imagine that in the concentration ratio of PLP/target ≤ 1
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46 there would be high probability for attachment of only one PLP molecule to one target molecule
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48 (Reaction 1). But at higher concentration of PLP (PLP/target > 1) there would be the possibility
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50 that each PLP molecule bind to more than one target molecule (Reaction 2). In such a case it
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52 prevents the circularization process.
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As shown in Fig. 2 (Inset), in the presence of constant concentration of target (0.20 pM), by increasing the PLP concentration up to 0.10 pM the signal reached a plateau then decreased as discussed earlier. Subsequently, a point between 0.10 and 0.25 pM was chosen as optimized PLP concentration (0.12 pM) throughout this research.

Optimization of ligation time

Optimization of ligation time (the time in which PLP is circularized) was performed at constant concentration of PLP (0.12 pM) and target (0.20 pM) by following the real time RCA reaction for 100 min. As shown in Fig. 3, by increasing the ligation time the signal also raised up. The maximum signal was achieved after 60 min then it reached a plateau (Fig. 3, Inset). Therefore, 60 min was chosen as optimum ligation time throughout this research.

Enzyme preference

So far different enzymes such as Phi29 DNA polymerase, Sequenase, Klenow, Vent exo-enzymes, and the BstI DNA polymerase were used for RCA reaction.²⁰ Among them Phi29 DNA polymerase^{20,25,38} and BstI DNA polymerase^{20,40,41,44} are most popular. Although, BstI DNA polymerase has mostly been used in HRCA reaction,^{14,27-29,35,40-43} we preferred to use Phi29 DNA polymerase due to its important advantages: A) it is able to perform the polymerization

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3 reaction at moderate temperature (30-37 °C).^{30,45,46} B) It is highly progressive so that the
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5 amplification reaction continues up to 70,000 nucleotides while it still is joining to the DNA
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7 template. The synthesis rate was reported to be ~1500 nucleotides per min.^{45,47,48} This, of course,
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9 could improve the sensitivity of the assay. C) Phi29 DNA polymerase degrades target from 3' to
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11 5' then it uses the 3' as a primer for RCA reaction which called target-primed RCA.^{49,50} So,
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13 contrasting to BstI DNA polymerase, Phi29 DNA polymerase doesn't need to use exonuclease
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15 enzyme prior to amplification reaction.
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20 21 *Calibration curve for H₅N₁*

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24 In view of the fact that influenza viruses are variant, it was important to evaluate the
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26 performance of biosensor in the presence of real H₅N₁ samples that may contain possible single
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28 nucleotide polymorphism. Therefore, in order to prepare the calibration curve, seven different
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30 real H₅N₁ samples were obtained from Tehran Veterinary Institute (Tehran, Iran) and their
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32 concentrations were determined by conventional real-time PCR (Fig. 4, A). Thereafter, the RCA
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34 signal for the standard real H₅N₁ samples were recorded against time. As seen, by increasing the
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36 concentration of target the signal intensity increased drastically over time and finally reached to a
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38 plateau (Fig. 4, A). Since, the signal at 88 min was more reproducible and linear (Fig. 4, B) this
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40 time was selected as fluorescence signal end point value.
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46 Finally, the calibration curve for real H₅N₁ targets was plotted by recording the RCA signal
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48 at different concentration of target at 88 min. As seen, using this procedure H₅N₁ was monitored
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50 in the concentration range from 10 fM to 0.25 pM (Fig. 4, B). The detection limit (DL) at signal
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52 to noise ratio of 3 (S/N = 3) was calculated according to the Eq. 1:
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$$56 \quad DL = 3.3 \sigma/S \quad (1)$$

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Where, σ is standard deviation of the response and S is the slope of calibration curve.⁵¹

The regression equation for H_5N_1 calibration curve was: $I = 252.8 [H_5N_1] - 36.59$ ($R = 0.993$, $n = 3$), and the relative standard deviation of the biosensor response at the concentration of 0.03 pM was 0.70% for three successive measurements. Based on Eq. 1, the detection limit was calculated to be as low as 9 fM, at signal/noise ratio of 3.

As seen in Fig. 5, at zero concentration of H_5N_1 background of about 30% fluorescence intensity was observed. To understand the source of this background it worth to note that in the absence of H_5N_1 the PLP could not be circularized however it can be hybridized with the forward primer (to recognize this sequence compare the PLP and forward primer sequences in Table 1) and form a double stranded oligonucleotide with 47 base pairs at most. Consequently, SYBR green intercalate in the oligonucleotide and produce a limited signal as background. It worth to note that in order to check the repeatability of biosensor responses each measurement was repeated three times and the results were reported as error bars in the figures.

As compared in Table 2, the analytical parameters such as detection limit and linear range obtained by the present method were much improved relative to those parameters reported in the literature.^{30,52-55} The reason for such improvements could be attributed to the nature of the amplifying process in HRCA. Using this process it is possible to generate more than 10^9 copies of each circle which results a huge signal amplification via DNA ramification.²⁵ The possibility for following the RCA in real-time, and the usage of sensitive fluorescence plate reader device as detector are two other advantages which provides better sensitivity and detection limit for H_5N_1 assay. Application of PLP is another advantage of this system which led to recognize the target molecules very efficiently via completion of hybridization of 3' and 5' end of the PLP to the target and ligation of the circular probe from head to tail^{56,57} as shown in Scheme 1. More

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3 improvement in this system was obtained by replacement of pH shock instead of heat shock.
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5 This not only makes the method simpler but also preserves DNA structure from potential
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7 breaking. In addition, it helps whole procedure to be carried out at low isothermal temperature.
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9 This is the reason we were able to improve the detection limit down to fM level. It is also
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11 important to note that this biosensor is label free which makes it much simple. Consequently, on
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13 the one hand the replacement of heat shock by pH shock simplify the system and improves the
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15 sensitivity and on the other hand escaping from labeling process, make the method easy to setup.
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17 It seems that these advantages help the system to be more applicable for commercial purposes.
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23 *Specificity*

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26 Specificity is an important factor in analyzing biological complex matrix. In this study, the
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28 specificity of the method was determined by comparing the signal obtained for H₅N₁ as target
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30 and hepatitis B virus (HBV), genomic DNA, H₇N₇ and H₃N₈ as unspecific target. A significant
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32 signal was observed for H₅N₁ while the signals for blank (negative control), HBV, genomic
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34 DNA, and two subtypes of influenza A virus that have been found in birds including H₇N₇ and
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36 H₃N₈ were the same as background (Fig. 5). In view of the fact that the target was designed
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38 based on the most specific and conserved part of H₅N₁ HA gene and due to accurate
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40 hybridization and circulization, PLP was able to detect H₅N₁ virus very specifically (Fig. 5).
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46 In addition, in order to prove the selectivity of PLP toward H₅N₁, both specific arms of PLP
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48 were aligned with segment 4 of H₃N₈ and H₇N₇ genomes (which encode HA gene in all subtypes
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50 of influenza A virus) by utilizing basic local alignment search tool (BLAST) software
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52 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The maximum score obtained for the alignment of the
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54 specific arms with HA genes of H₃N₈ and H₇N₇, was 16.4 which is too low to be specifically
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3 detected. Therefore, the PLP cannot be circularized to be used as a template in RCA reaction. It
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5 is also noticeable that influenza A viruses exchange their genomic segments via the mechanism
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7 of the antigenic shift which occurs when two different flu strains combine and infect the same
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9 cell. Therefore, it is possible for the genomic segments to be transferred from one strain to
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11 another one. According to the sequences provided by genomic database
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13 (www.ncbi.nlm.nih.gov), the recognition region for the proposed PLP also exists in H₅N₅
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15 subtype; however, the frequency of the reported target is too low (one out of 100, sequence ID:
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17 [gb|JX878683.1](http://www.ncbi.nlm.nih.gov/nuclseq/gb|JX878683.1)). Thus, the veracity of this report is doubtful.
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23 *Feasibility for real samples*

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27 In order to investigate the feasibility of the proposed biosensor for determination of H₅N₁ in
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29 real sample, seven specimens obtained from the sick birds were examined. At first, the
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31 concentration of H₅N₁ in real positive samples was determined to be 0.18, 0.14, 0.14, 0.19, 0.18,
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33 0.14 and 0.18 pM by using standard real-time PCR. Then, the fluorescence intensities of these
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35 samples were recorded by the proposed biosensor. Based on the calibration curve reported in Fig.
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37 4, B the concentration of H₅N₁ in real positive samples was determined to be 0.16 ± 0.002 , 0.13
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39 ± 0.004 , 0.12 ± 0.006 , 0.17 ± 0.003 , 0.15 ± 0.004 , 0.13 ± 0.005 and 0.17 ± 0.004 pM,
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42 respectively (Table 3). As seen there is a satisfactory consistency between the data obtained by
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44 standard real-time PCR and the RCA based biosensor. This indicates the feasibility of the
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46 proposed biosensor for the determination of H₅N₁ in clinical analysis.
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51 **Experimental**

52 **Chemicals**

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3 The length of PLP, used in this assay, was 112 nucleotides. This circularizable
4 oligonucleotide probe was consisting of two adjacent complementary sequences which were
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6 chosen from the most conservative parts of the H₅N₁ HA gene by utilizing the BioEdit Sequence
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8 Alignment Editor software (19 and 22 nt). Also it has a linker region (71 nt) to facilitate
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10 formation of circular template and provide spaces for RCA primers binding. Since the target
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12 sequence was highly identical for this subtype, it can only detect H₅N₁ subtype of influenza A
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14 virus.^{1,58,59} Furthermore, conservation of the PLP binding site was evaluated by utilizing BLAST
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16 for analyzing of the sequence. After performing BLAST, recognition site for PLP was 100
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18 percent identical in all HA genes of H₅N₁ virus that were reported in genomic database. It means
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20 that, this PLP has capability to detect all variants of H₅N₁ virus.
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27 The 5' phosphorylated linear PLP and oligonucleotides (synthetic target and 3' phosphoro-
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29 thioate modified primers) were purchased from Metabion (Martinsried, Germany). The primers,
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31 probe and synthetic target are listed in Table 1. The RevertAid First Strand cDNA Synthesis Kit,
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33 T₄ ligase buffer, T₄ ligase enzyme, Phi29 buffer, Phi29 DNA polymerase, deoxyribonucleotide
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35 triphosphate (dNTP) and SYBR green I were obtained from Fermentas (USA). KOH and HCl
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37 were purchased from Merck (Germany). Ethylenediaminetetraacetic acid (EDTA), Tris-HCl,
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39 agaros, Tris-acetate and ethidium bromide were purchased from Sigma-Aldrich (USA). RNA
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41 extraction kit, real-time PCR kit and 384 well plates were respectively obtained from Jena
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43 Bioscience (Germany), Genesig (United Kingdom) and Greiner Bio-One (Germany). In all
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45 experiments the solutions were prepared by utilizing double distilled de-ionized water. And all
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47 chemicals were used without further purification.
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54 Apparatus

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Virus cDNA was synthesized in Eppendorf thermo cycler (Germany). Electrophoresis was performed in Akhtarian electrophoresis tank (Tehran, Iran) and voltage was adjusted by Bio-rad power supply (California, USA). The gel was visualized by SynGen transilluminator (United Kingdom). The fluorescence emissions were recorded at 520 nm by fluorescence micro plate reader (H4, Bio Tech Co, USA). Real-time PCR has been carried out by using Rotor-Gene Qiagen real-time PCR cycler (USA).

Virus samples and cDNA synthesis

The infected samples were obtained from the sick birds. RNA was extracted using RNA extraction kit. And cDNA was synthesized by RevertAid First Strand cDNA Synthesis Kit which temperature was adjusted in thermo cycler.

PLP ligation and hyperbranched rolling circle amplification

One μl of cDNA of H_5N_1 virus, 1 μl of PLP and 1.2 μl of denaturing solution (pH 12) containing 400 mM KOH and 10 mM EDTA were mixed (by pipetting the mixture up and down) and incubated for 3 min at room temperature. Then, 0.8 μl of neutralizing buffer containing 600 mM Tris-HCl and 400 mM HCl was injected in to the reaction mixture gradually (If this process carries out precisely the exact final pH will be 7.9). Afterward, 6 μl of the ligation mixture containing one μl of $10\times$ T_4 ligase buffer (consisting of 400 mM Tris-HCl, pH 7.9, 100 mM MgCl_2 , 100 mM DTT, 5 mM ATP) and 5 μl deionized water was added to the reaction mixture. The ligation reaction was done by addition of 2 units (U) of T_4 ligase to the mixture and incubating at 37 °C for 60 min. The ligation reactions were terminated by heating at 70 °C for 5 min. Thereafter, 40 μl of RCA mixture containing final concentration of $1\times$ Phi29 buffer (33 mM Tris-acetate (pH 7.9), 10 mM Mg-acetate, 66 mM K-acetate, 1% (v/v) Tween 20, 1 mM DTT),

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3 500 μM dNTP, 10 pM forward primer, 10 pM reverse primer, 8 U of Phi29 DNA polymerase
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5 and 1 \times SYBR green I was added to 10 μl ligation reaction mixtures. The mixture (50 μl) was
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7 poured in a well. The plate was placed in fluorescence plate reader device and the real-time
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9 fluorescence intensity was recorded for 100 min at 37 $^{\circ}\text{C}$. To stop the reactions then the mixture
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11 was incubated at 65 $^{\circ}\text{C}$ for 10 min and finally stored at 4 $^{\circ}\text{C}$. Therefore, the total time takes to
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13 run the RCA technique for H₅N₁ assay (Scheme 1) was about 180 min.
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17 18 *Gel electrophoresis assay*

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21 Electroforetic analysis of PLP, C-PLP (circular padlock probe) and HRCA products was
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23 performed in 1% agarose gels filled with TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH
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25 8.0). This process carried out for 30 min and under the applied voltage of 100 V. Then the gel
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27 was stained by ethidium bromide and visualized by transilluminator at 302 nm.²⁴
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31 32 *Real sample detection*

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35 The designed RCA technique was evaluated by quantification of seven real samples. The
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37 results were compared with those obtained by the standard method of real-time PCR using a real-
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39 time PCR kit. It is important to note that the H₅N₁ specific primers that amplified by PCR
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41 contained about 130 bases within conserved portion of H₅N₁ HA gene.
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45 46 **Conclusion**

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49 The detection limit (9 fM) and linear range (10 fM to 0.25 pM) obtained by the present
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51 isothermal amplification technique brought us to the conclusion that integrating of PLP, HRCA,
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53 Phi29 DNA polymerase and pH shock process much improved the sensitivity, specificity and
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55 linearity of the RCA based biosensor toward H₅N₁ virus, relative to those methods reported in the
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3 literature. Comparing to the conventional PCR technique, the method showed some advantages
4 such as isothermal amplification and higher sensitivity. Also comparison between the data
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6 obtained by standard real-time PCR and the RCA based biosensor proved the reliability of the
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8 method. In addition, by applying Phi29 DNA polymerase and pH shock process the HRCA
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10 reaction could be performed at moderate temperature. Therefore, it seems that the proposed
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12 biosensor has potential to be used as a user friendly DNA diagnostic method for the
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14 determination of H₅N₁ in clinical analysis.
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20 **Acknowledgements**

21
22 The financial support provided by the Research Council of the University of Tehran and the
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24 Tehran University of Medical Sciences is acknowledged.
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Figure captions

Scheme. 1: Representation of the RCA technique for H_5N_1 assay. a: linear PLP (L-PLP), b: H_5N_1 target, c: T_4 DNA ligase enzyme, d: Phi29 DNA polymerase, e: nucleotides, f: SYBR green. I: Combination of denaturation solution with PLP and H_5N_1 target was followed by gradually addition of neutralizing solution in order to hybridize PLP with H_5N_1 . II: The ligation step for 60 min at 37 °C. During this process DNA ligase seals the adjacent 5' and 3' ends of PLP, thereby creating a circular molecule. III & IV: Phi29 DNA polymerase degrades H_5N_1 from the direction of 3' to 5' then uses 3' end as template for initiation of RCA reaction. V & VI: Circularized probes are amplified by HRCA reaction. This results a long stretch of concatemer products. As the reaction is precede the fluorescence molecules are intercalated in the produced double strand DNA. This process can be detected by measurement of relative fluorescence intensity in a time course of 100 min at 37 °C.

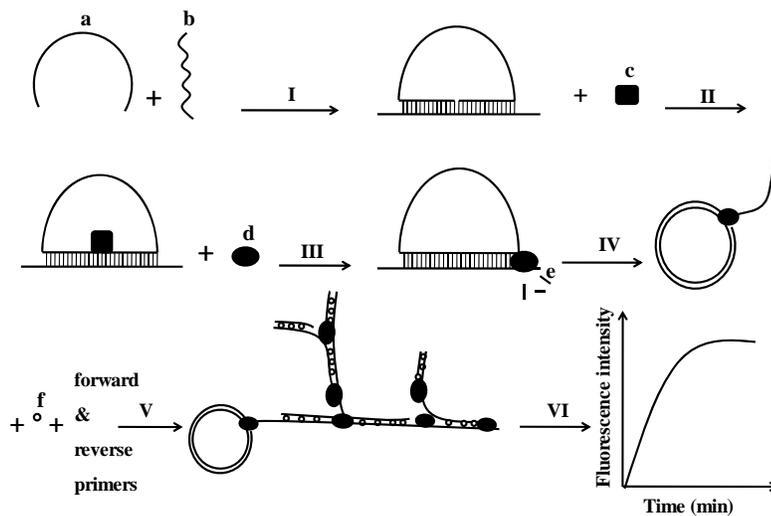
Fig. 1: Gel electrophoresis for representation of PLPs (linear and circular) and HRCA product. A) Linear and circular forms of PLP made from 112 nucleotides single strand DNA. Lane M: markers. Lane 1: linear-PLP. Lane 2: circular PLP. B) Analysis of HRCA product. Lane 1: blank sample (without any target molecule). Lanes 2 and 3: HRCA product. The electrophoresis was carried out using 1% agarose gel (20 cm length) for 30 min and under the applied voltage of 100 V.

Fig. 2: Optimization of PLP concentration for H_5N_1 assay. The HRCA reaction was carried out at 37 °C in the presence of constant concentration of target (0.20 pM) and different concentrations of PLP, from down to up: 0.01, 0.05, 0.50, 0.10 and 0.25 pM.

Fig. 3: Fluorescence intensity against time. Ligation reactions were carried out at different times of 15, 30, 60 and 90 min and at 37 °C, in the presence of 0.20 pM H_5N_1 target and 0.12 pM of PLP. Then the fluorescence intensity was recorded via real-time RCA. Inset shows the optimum ligation time obtained by comparing the fluorescence intensities at plateau.

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4 Fig. 4: (A) Fluorescence intensity against time at different concentrations of real H₅N₁ samples. Real-time
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6 RCA signal was recorded at different H₅N₁ real samples concentrations, from down to up: 0, 0.01, 0.03,
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8 0.06, 0.12, 0.20, 0.40 and 0.25 pM at 37 °C. (B) The calibration curve for real H₅N₁ samples.
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11 Fig. 5: Comparison of specificity of HRCA biosensor towards different DNA samples. Real-time HRCA
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13 signal was recorded toward different synthetic molecules of H₅N₁, genomic DNA, HBV, H₇N₇, H₃N₈ and
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15 blank as negative control. All samples were used at 0.25 pM concentration. The experiments were carried
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17 out at 37 °C and PLP concentration of 0.12 pM.
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Scheme. 1

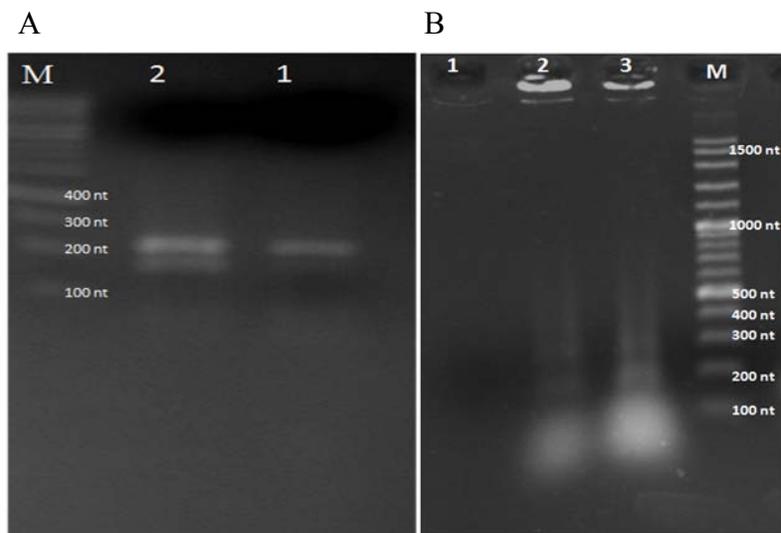


Fig. 1

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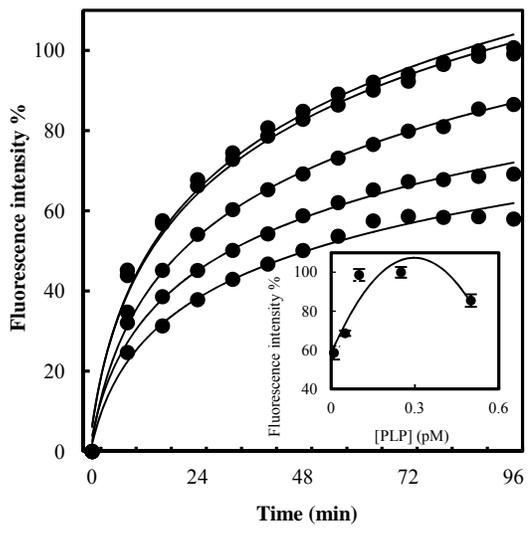


Fig. 2

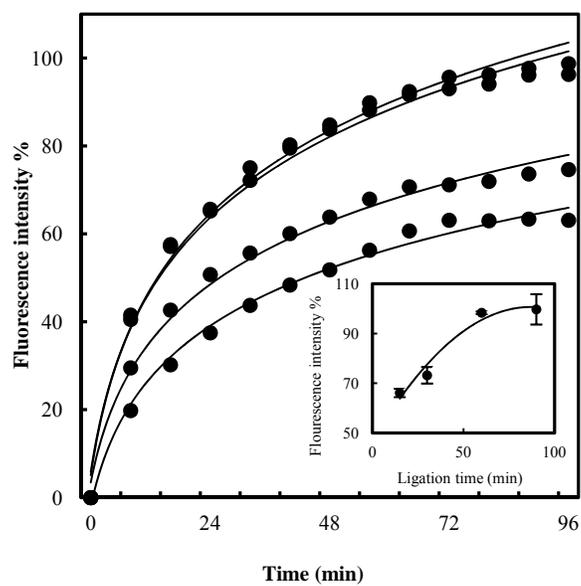


Fig. 3

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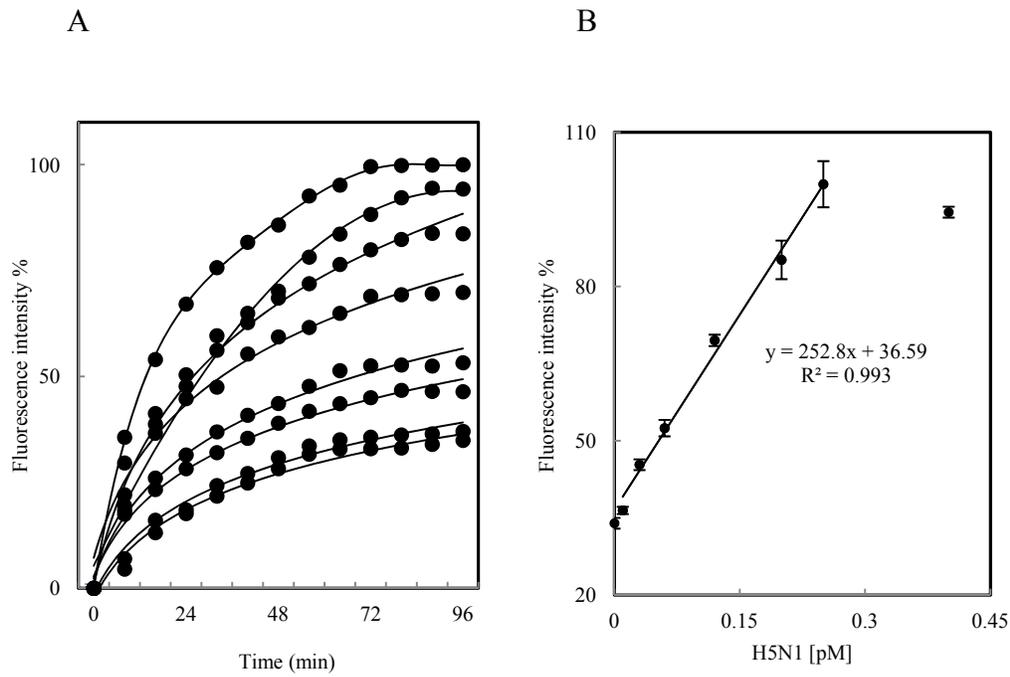


Fig. 4

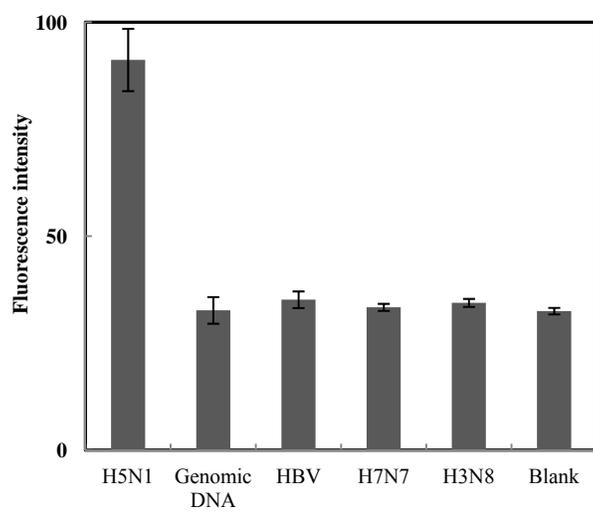


Fig. 5

Table 1

The sequences for PLP, primers and target

Type of oligonucleotides	Sequence
PLP [†]	5' P-GGATGATCTGAATTTTCTCAAACCCGGTCAACTTCAAGCTCCTAAGCC TTGACGAACCGCTTTCCTGACTGAATGCAGCGTAGGTATCGACTTCG GACCAAGAACTTTTGG-3'
Forward primer	5'-GCTTAGGAGCTTGAAGTTG*A*C-3'
Reverse primer	5'-GCTTTGCCTGACTGAATGC*A*G-3'
H ₅ N ₁ target	5'-TTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCGA-3'

P at 5'-end of probe indicates phosphorylation.

The stars (*) at 3'-end of primers indicate phosphoro-thioate modifications.

Table 2

Comparison of analytical parameters obtained by RCA method for different targets.

Target	Detection method	Linear range (pM)	Detection limit (pM)	References
H ₃ N ₁ gene	Fluorescence	0.01-0.25	0.009	Present work
β-thalassemia gene	Ultraviolet (UV)	0.30-80	0.07	52
PDGF	Fluorescence	300-80000	300	30
Streptavidin	Electrochemical	1-500000	0.4	53
Epididymis-specific protein 4	Electrochemical	3–300	0.06	54
PDGF	Electrochemical	84-8400	63	55

Table 3

The biosensor responses for determination of H₅N₁ in real bird serum sample. Before measurements, these samples were controlled by conventional real-time PCR technique.

Methods	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
	(pM)						
Present biosensor	0.16 ± 0.002	0.13 ± 0.004	0.12 ± 0.006	0.17 ± 0.003	0.15 ± 0.004	0.13 ± 0.005	0.17 ± 0.004
Real-time PCR	0.18	0.14	0.14	0.19	0.18	0.14	0.18