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Naked Eye Y Amelogenin Gene Fragment Detection Using DNAzymes on a Paper-based Device

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ABSTRACT

Nowadays, there are strong efforts in developing new technology for rapid detection of specific DNA sequences for environmental monitoring, forensic analysis and rapid biomedical diagnosis applications. That is where microfluidic paper-based analytical devices are positioned as suitable platforms for the development of point of care analytical devices, due to their simple fabrication protocols, ease of use and low cost. Herein, a methodology for *in situ* single strand DNA detection by using a colorimetric assay based on the formation of a DNAzyme within a paper substrate was developed. A DNAzyme that could only be formed in the presence of a specific sequence of the Y human amelogenin gene was designed. The performance of the DNAzyme was followed colorimetrically first in solution and then in paper substrates. The reaction was found to be specific to the Y fragment selected as analyte. The DNAzyme reaction on paper enabled the unequivocal colorimetric identification of the Y single strand DNA fragment both qualitatively, with the naked eye (143 ng), and quantitatively by image analysis (45.7 ng). As a proof of concept, a microfluidic paper-based device, pre-loaded with all DNAzyme reagents, was characterized and implemented for the simultaneous detection of X and Y single strand DNA fragments.

Keywords: DNAzyme, amelogenin, paper substrate, gene detection, colorimetric, paper based microfluidics.

1. INTRODUCTION

There is an increasing interest in producing new technology for fast detection of specific DNA sequences at the point of need for forensic analysis, environmental monitoring and rapid biomedical diagnosis applications. Widely known methodologies such as qPCR or PCR-capillary electrophoresis are mainly based on the amplification of DNA samples to achieve enough quantity of DNA to be analyzed [1]. These methods require specialized procedures, equipment and personnel to carry out the analysis.

On the other hand, point of care tests (POC) [2] refer to devices that analyze, with minimum intervention of the user, small volume of samples at the point of necessity in short times. Moreover, they are usually designed to be mass-fabricated at low costs [3]. Microfluidic paper-based analytical devices (μPADs) constitute suitable platforms for the development of POC systems since they fulfil all the previously described characteristics [4,5]. Several paper-based types of materials, such as cellulose and nitrocellulose, can be easily adapted to serve as μPADs [6–8]. Due to their fibers distribution, liquid can flow inside of the material by imbibition, enabling the movement of liquids through its matrix without the requirement of external pumps [9]. Wax-printing, which involves the generation of hydrophobic walls within the paper matrix by printing and melting of wax, is proposed as the best methodology for the fabrication of μPADs due to the easiness of the procedure and the capacity to be escalated to mass-production [8,10]. Furthermore, μPADs are disposable, becoming a more environmentally friendly option than other microfluidic devices [11]. A number of μPADs including, HIV Tests (Home Access, USA), paper ELISA, and low-cost colorimetric diagnostic assays have been already described [12,13] and commercialized.

Traditionally, available paper-based devices are centered on the detection of big molecules such as proteins. Nevertheless, the development of novel μPADs for nucleic acid detection, such as nucleic acids capture on cellulose filter paper with *in situ* PCR [14], loop-mediated isothermal amplification of

several DNA targets on paper device[15] and colorimetric DNA detection through gold nanoparticles capture and agglomeration in paper device[16], among others, is a hot topic at the moment.

DNAzyme is composed of a DNA oligonucleotide, capable of performing a chemical reaction, often a catalytic reaction. In particular, DNAzymes sensing probes based on G-rich sequences, known as G-quadruplex, are gathering special attention due to their simplicity, high sensitivity and selectivity for the recognition of small molecules [17,18]. The G-quadruplex creates a special hairpin on the DNA sequences forming a complex with peroxidase activity, enabling the oxidation of substrates like 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in the presence of hydrogen peroxide (H_2O_2), producing an appreciable color change of the solution where dissolved [19–21]. By controlling the design of the DNAzyme sequence, it is possible to specifically detect ribonucleic acid (RNA), DNA, proteins and metal ions [22,23]. In this regards, designing a DNAzyme able to hybridize to a single strand DNA (ssDNA) sequence allows for the specific detection of the sequence of interest [24–26].

The use of DNAzymes in paper substrates was recently presented for the design of novel analytical devices. Zhang Y. *et al.* [27] demonstrated that hemin/G-quadruplex DNAzymes can be used on a paper support for the detection of potassium cations. Monsur Ali M. *et al* [28] achieved the genetic detection of *Escherichia coli* RNA on a paper-based filter support using DNAzyme catalytic reactions. However, their detection method was based on fluorescence, which required of specific instrumentation, thus increasing the complexity and cost of the methodology, whereas a colorimetric detection could be a better choice to make [29].

Here, a colorimetric methodology for the detection of ssDNA sequences by specific DNAzymes in solution, a Whatman filter paper 1 support and a paper-based device is presented, Figure 1. The formation of the DNAzyme in the presence of the analyte allows the oxidation of ABTS producing a green color, $ABTS^+$, which is detected with the naked eye and quantitatively, using colorimetric image analysis from a mobile camera. As a proof of concept, a synthetic fragment corresponding to

Y amelogenin gene, which differs from its X counterpart by 6 base pairs, was designed as a model to test ssDNA detection [30–32].

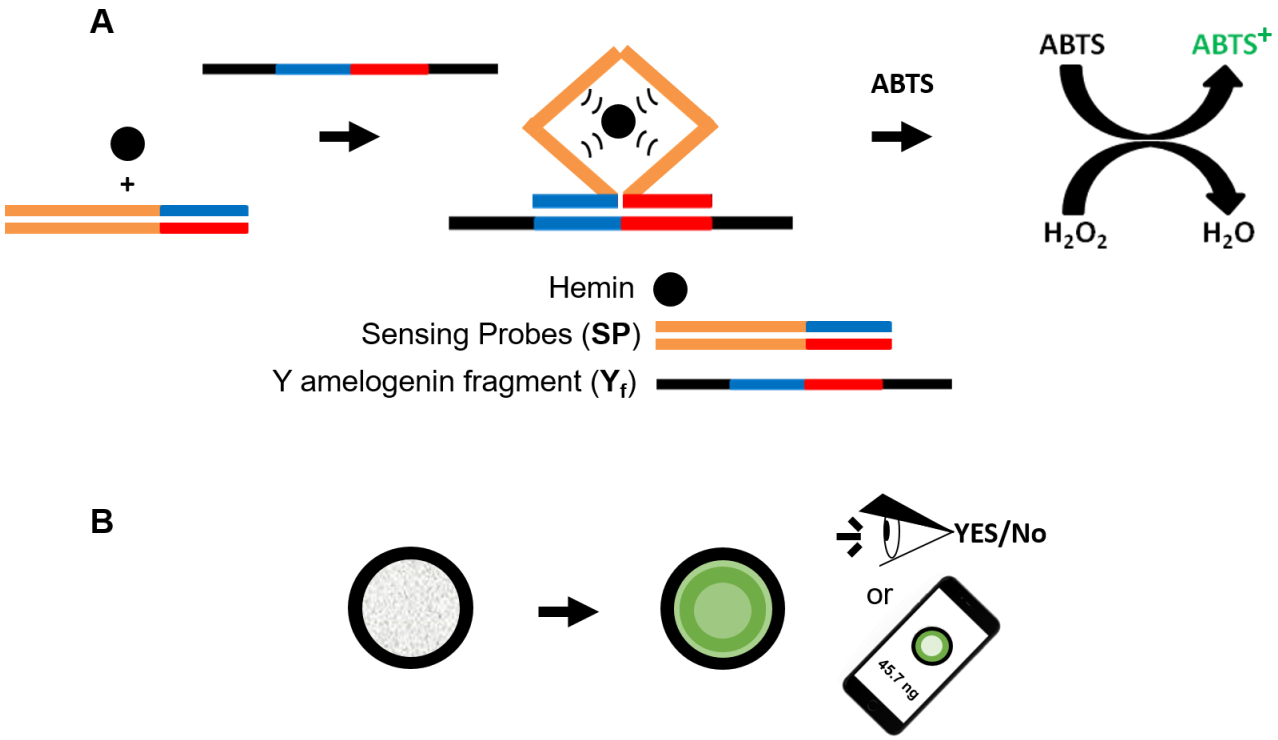

Figure 1. Schematic representation of the DNAzyme formation and specific Y amelogenin fragment (Yf) identification of paper support. A) DNA sensing probes and hemin specifically bind to Yf to form a three dimensional structure with catalytic activity able to oxidize ABTS in the presence of H2O2, producing green colored ABTS+. B) DNAzyme reaction on paper support produces an appreciable green color in the presence of the Yf thanks to the oxidation of ABTS. The reaction can be monitored colorimetically.

2. EXPERIMENTAL

2.1. Reagents and Equipment

Oligonucleotides. DNA sequences (DNA-active, DNA-sensing probes and Y and X amelogenin fragments) were synthesized by Integrated DNA Technologies (IDT, Belgium).

Reagents. Hemin, ABTS, dimethyl sulfoxide (DMSO), HEPES buffer, sodium chloride, Triton X-100 and dimethylsulfoxide were purchased from Sigma-Aldrich (Spain). H_2O_2 was obtained from E. Merck (Germany). Potassium chloride was purchase from Panreac (Spain).

Equipment. *Solution assays* were performed in a microtiter plate CORNING 96 wells and colorimetric values were recorded with a Beckman Coulter DTX 880 Multimode Detector.

Paper assays were performed on Whatman filter paper 1(Sigma Aldrich, Spain) and Hi-Flow Plus HFC 12004 nitrocellulose (EMD Millipore, Ireland). The paper assays and the paper-based device were printed by a Xerox ColorQube 8570 wax printer and the wax barriers were generated with an oven, set at 125 ºC for 5 min.

Photographs were taken by a Sony Xperia Z3 D6603 mobile phone camera (20.7 MP, f/2.0, 25 mm (wide), 1/2.3", 1.12 µm, AF) or a LaserJet Pro 400 MFP scanner (Scanning Method: Flatbed; Scanner Type Flatbed: DF; Bit Depth: 30-bit).

Buffer solution: HEPES 2.5 10^{-2} M (Sigma-Aldrich, Spain), Potassium Chloride 2 10^{-2} M (Panreac, Spain), sodium chloride 0.2 M (Sigma-Aldrich, Spain), Triton X-100 0.05 % and dimethylsufoxide 1 % (Sigma-Aldrich, Spain), at pH 7.4 with sodium hidroxide (Sigma-Aldrich, Spain) in water, was freshly prepared in the laboratory.

2.2. DNAzyme performance in solution

For specific Y_f detection, 1 µL of a mix of DNA-sensing probes 1 and 2 (SP1 and SP2, Scheme 1) (100 µM each probe) and 47 µL of buffer solution were pipetted in the microtiter plate and incubated for 1 h. Then, 1 µL of hemin solution (100 µM) was added and incubated for 1 h. After that, 1 µL of either Y_f or X amelogenin fragment (X_f) solutions ranging from 50 to 150 µM were added and incubated for 30 min (n = 3). Finally, 25 µL ABTS solution (28.8 mM) and 25 µL H_2O_2 solution (1.76 mM) were pipetted. The concentrations were previously optimized for this reaction, see supporting information SI-1, as well as the type of probe used for the assay, see supporting information SI-2.

The absorbance was measured at 415 nm every 5 min for 30 min using a Beckman Coulter DTX 880 Multimode Detector.

Y amelogenin fragment (Y_f)	CCCTGGGCTCTGTAAAGAATAGTGGGTGGATTCTTCATCCCAAATAAAGTGGTTTCTCAAGTGGTCCCAATTTT
X amelogenin fragment (X_f)	CCCTGGGCTCTGTAAAGAATAGTGTGTTGATTCTTTATCCCAGATGTTTCTCAAGTGGTCCTGATTTTACAGTTCC
DNA-active	TTTGGGTAGGGCGGGTAGGG
DNA-sensing probe 1 (SP1)	CGGGATGGGTTTTTGGGATGAAGAATCCA
DNA-sensing probe 2 (SP2)	GAAACCACTTTATAAAGGGTAGGG

Scheme 1 DNA sequences: Y_f, X_f, DNA-active and sensing probes 1 and 2 (SP1 and SP2) used in this study.

2.3. Y amelogenin fragment discrimination and analysis in paper substrate

The Y_f detection through DNAzyme was carried out in a paper substrate. Therefore, circle-shaped structures (1 cm, inner diameter after heating) were wax-printed to set the sensing areas in the two paper supports. After printing, the circles were heated in the oven for 5 min at 125 °C. The heating step is necessary in order to melt the wax trough the paper and generate the hydrophobic barriers [8,10].

Determination of the most adequate paper substrate: Whatman filter paper 1 or nitrocellulose membrane

In order to test the most suitable type of paper for Y_f detection, the oxidation of ABTS with a DNAzyme-active solution, serving as a control, was carried out in Whatman filter paper 1 and nitrocellulose membrane. First, 1 cm wax-circles were printed on both type of substrates. Then, 0.5 µL of DNA-active (100 µM), 0.5 µL of hemin (100 µM) and 24 µL of buffer solutions were pipetted and incubated for 1 h, at room temperature (RT). The concentrations of DNA-active and hemin were previously optimized for this reaction in solution, see supporting information SI-1. Finally, 12.5 µL of ABTS solution (28.8 mM) and 12.5 µL of H_2O_2 solution (1.76 mM) were added in order to generate the color assay. The concentrations of ABTS/H_2O_2 were previously optimized for this reaction in

142 solution, see Supporting Information SI-1. Photos were taken with a mobile phone camera before the
143 addition of ABTS and H_2O_2 solutions and 5 and 60 min after addition.
144
145 *Detection and quantification of Y_f on the paper substrate: Whatman filter paper 1*.
146 0.5 cm wax circles (inner circle, after melting) were wax-printed. 0.25 μL of a 1:1 mix of SP1 and
147 SP2 (100 μM each), 0.25 μL of hemin-stock solution (100 μM), 6 μL of ABTS-stock solution (40
148 mM) and 7 μL of buffer solution were mixed in an Eppendorf. The resulting mixture was pipetted on
149 the paper wax-circle. After overnight storage at 4 ºC, either 0.25 μL of Y_f (25, 50, 100 or 200 μM),
150 X_f (25, 50, 100 or 200 μM) or buffer solution were pipetted in the wax-circles (n = 3) and 30 min
151 incubated at RT. Then, 6 μL of H_2O_2 (2.4 mM) was pipetted in all wax-circles. Images were taken by
152 a mobile phone camera after 5 min.
153
154 *Detection and quantification of Y_f on the paper substrate in the paper-based device*
155 For the specific detection of Y_f fragment in the paper-based device (See Supporting Information SI-
156 3, for device specifications). An optimized mixture of the reagents, ABTS and H_2O_2 were used for
157 these experiments, see supporting information SI-1 and SI-2.
158 0.25 μL of a 1:1 mix of SP1 and SP2 (100 μM), 0.25 μL of hemin-stock solution (100 μM), 6 μL of
159 ABTS-stock solution (21.6 mM) and 7 μL of buffer solution were pipetted in the device reservoirs
160 (Y and X wax-circles,), in three different devices. The mix of this reagents forms the Y_f-DNAzyme
161 cocktail. After overnight storage at 4 ºC, 0.25 μL of Y_f and 0.25 μL of X_f (0.5 μM) were pipetted on
162 their respective detection zones and incubated for 3 min at RT. Finally, 12 μL of H_2O_2 solution (1.32
163 mM) was pipetted on the inlet (center of the device) and the solution was left to run until it reached
164 both detection zones. Photographs were taken by the mobile phone camera 5 min after H_2O_2 solution
165 reached the detection zones.

2.4. Image and data analysis

All images were transformed to 8-bit and grey intensities (corresponding to the total color intensity [12,33]). They were analyzed using Image-J software. Paper background intensity was subtracted from all data. Data plotting and statistical analysis were carried out in Excel and Origin Pro 2018.

3. RESULTS AND DISCUSSION

In order to investigate the use of DNAzymes on paper substrates for naked eye detection of ssDNA fragments and quantification by image analysis with a mobile camera, a DNAzyme for the detection of a specific fragment, Y_f, of the Y amelogenin gene was designed. Then, a reaction cocktail comprised of hemin, ABTS and two sensing probes (SP1 and SP2, which bind specifically to Y_f) was developed; see supportive information SI-1 and SI-2 for detailed information of the characterization of the different components of the cocktail.

To evaluate the sensitivity and selectivity of the designed DNAzyme assay for the detection of Y_f *versus* X_f, the DNAzyme cocktail was incubated with different concentrations of Y_f and X_f ranging from 0.5 to 1.5 μM (corresponding to a range between 1000 and 3000 ng of ssDNA per sample), and the absorbance of the final solutions were measured in a conventional microtiter plate reader after 5 min of incubation. An increase in absorbance was appreciated over time and when increasing the concentration of Y_f. On the other hand, the absorbance values for the reaction performed with the X_f remained constant over time and for all concentrations investigated, Figure 2. This results demonstrate the specific reaction of the DNAzyme with the Y_f fragment, while the same DNAzyme was not able to detect the X_f fragment, in solution.

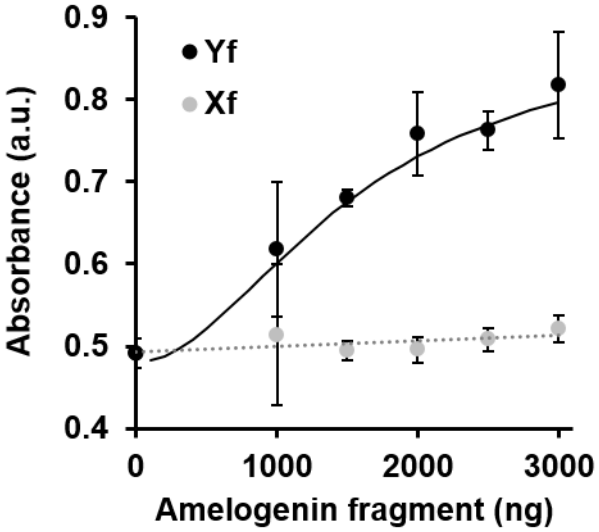

Figure 2. Graphical representation of Y_f detection in solution. Y axis indicates the absorbance obtained from the oxidation of ABTS with different amounts of Y_f and X_f at 5 min. Fitting curve of Y_f was done through the equation $y = 0.89-(0.41/(1+(x/1.58)^{1.89}))$, $R^2 = 0.9909$. Error bars mean ± SD (n = 3 samples per experimental condition).

Y_f absorbance values were fitted to a 4 parametric logistic (4PL) curve, which was used before in immunoassays and binding ligand assays [34]. A limit of detection (LoD) of 655 ng of Y_f was calculated following the equation 1,

$$LoD = mean_{blank} + 3\,SD_{blank} \quad \text{(eq. 1)}$$

where $mean_{blank}$ is the absorbance value of the reaction mix without Y_f and X_f , and *SD* is the standard deviation (n = 3) of the mean value of the blank. And a limit of quantification (LoQ) of 1462 ng of Y_f was calculated following the equation 2,

$$LoQ = mean_{blank} + 10\,SD_{blank} \quad \text{(eq. 2)}$$

where $mean_{blank}$ is the absorbance value of the reaction mix without Y_f and X_f , and *SD* is the standard deviation (n = 3) of the mean value of the blank. High *SD* values were observed at low amounts of Y_f and X_f, (1000 ng). This higher *SD* corresponds to the experimental error, which comes from the number of dilutions needed to reach this 1000 ng solution from the X_f and X_f main stock solution.

This error could be reduced by increasing the number of experiments per concentration. Therefore, the calculated LoQ value was taken as more certain value in the analysis of the dynamic range of the reaction. The dynamic detection range (1462 – 3000 ng) is limited by the increasing signal of the background over time, provided by the spontaneous ABTS oxidation in the reaction mix, as previously described in literature [35,36].

3.2. Selectivity and Sensitivity of the assay in the Whatman filter paper substrate

In order to get an assay that is suitable for a paper-based device configuration, it is necessary that all the reagents of the assay are paper compatible, can be preloaded and, if possible, stored for long periods of time and at different ambient conditions (*e.g.* light and temperature). Therefore, the stability of the assay over time, was investigated for two light conditions (daylight and dark at RT) as well as at 4 ºC in the dark for 24 h storage; see Supporting Information SI-4. The absorbance values of the assay in solution were lower than those carried out without storing for 24 h, but they clearly allow the detection of the Y_f in the same range of concentrations and times than when no storage was carried out. Therefore, these protocols can be implemented during the fabrication of a μPAD, with all the reagents preloaded and stored in paper.

The assay solutions stored at daylight and at RT for 24 h did not significantly have an increase in color due to ABTS oxidation, see section Figure SI-4 B and C. Moreover, the assay kept at 4 ºC in the dark presented higher absorbance values due to a better preservation of the reagents at low temperature, see Figure SI-4 D, being this protocol (4 ºC in the dark) the one used in experiments hereafter.

After confirming the possibility to detect Y_f using the designed DNAzyme in solution, the conditions to perform the detection of the assay on a paper substrate were investigated. Two different papers, nitrocellulose membrane and Whatman filter paper 1, which are commonly used for the fabrication of lateral flow assays and μPADs, were investigated. Using a wax printer, 1 cm diameter wax-circles

were printed on both types of substrates. Initial experiments on paper were performed using a positive control mix comprised of DNA-active sequence and hemin group to form a DNAzyme without the presence of the target analyte (DNAzyme-control mix). First, 0.5 μL of DNAzyme-control mix and 24 μL of buffer solution were pipetted and incubated for 1 h at RT in the paper substrates. Then, 12.5 μL of ABTS solution (28.8 mM) and 12.5 μL H_2O_2 (1.76 mM) solution were added. Photos of the substrates were taken with a mobile phone camera 0, 5 and 60 min after addition of ABTS and H_2O_2 (Figure 3).

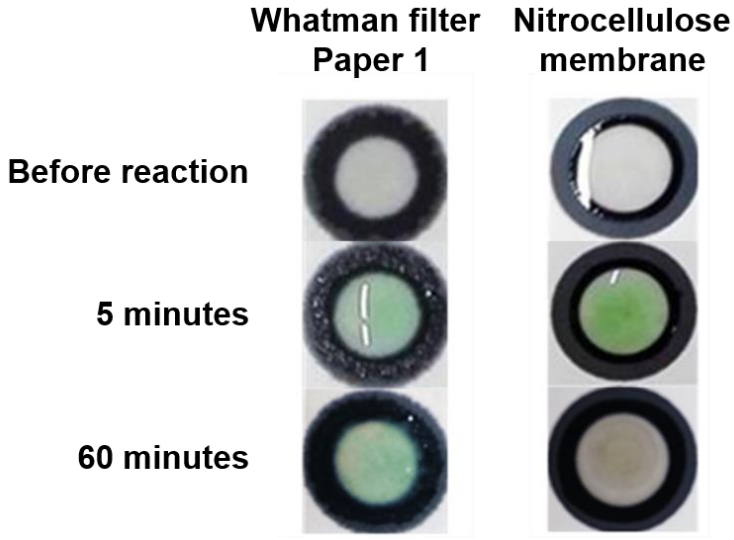

Figure 3. Pictures of DNAzyme control mix in Whatman filter paper 1 and nitrocellulose membrane. Photographs were taken at times 0, 5 and 60 min after ABTS/H_2O_2 solutions addition. Green color indicates the presence of $ABTS^+$.

The Whatman filter paper 1 presented a better performance than the nitrocellulose. The Whatman paper 1 had high ability to absorb the reagents and integrate them within the paper fibers, with the reaction taking place in the paper substrate. After the assay was carried out (addition of ABTS/ H_2O_2 solutions), the paper substrate kept the green color, which is the result of the enzymatic reaction, even after 60 min (dry paper). Considering this result it can be speculated that the fibers of the Whatman paper 1 were able to stabilize the $ABTS^+$ over time, up to 60 min.

On the other hand, the nitrocellulose paper substrate did not absorb the reagents, remaining in solution in the drop, held by the wax-printed-circle. After the assay was carried out (addition of ABTS/ H_2O_2 solutions), the oxidation reaction took place in solution and not in the fibers of the nitrocellulose paper. The oxidized $ABTS^+$ molecules (green color) were not stabilized by the paper substrate and continued the oxidation process. Under prolonged oxidative conditions, $ABTS^+$ suffers over-oxidation into $ABTS^{2+}$, which is not stable in aqueous solution and decompose into several uncolored by-products, losing its characteristic green color [37–39]. In view of these results, the Whatman filter paper was selected as the right support for the DNAzyme reaction.

The detection of Y_f on Whatman paper 1 was evaluated using the DNAzyme on a 2 by 5 array of 0.5 cm diameter (internal diameter, after heating) wax-printed circles. 12 μL of a solution containing 0.25 μL of the sequences SP1 and SP2 (1 μM) with 0.25 μL of hemin solution (1 μM), 6 μL of ABTS (40 mM) and 7 μL of buffer were pipetted and subsequently dry over night at 4 ^oC in the dark in each circle. Then, 0.25 μL of solutions with increasing concentrations of Y_f or X_f ranging from 0 (negative control) to 200 μM were loaded in each reaction circle and let dry for 30 min at RT. Finally, 6 μL of H_2O_2 (2.4 mM) were added to each reaction circle. After 5 min a green color was appreciated in all the reaction circles, by naked eye. A pronounced “coffee ring” effect was identified in all the circles containing Y_f, while this coffee ring was not appreciated in the circles containing X_f. The shape of the coffee ring corresponded with the shape of the drop of X_f previously loaded on the substrate. The reason for the formation of this ring can be explained by the concentration of the reagents during the drying process of the sample drop pipetted in the paper support [40]. Additionally, a darker green color intensity was appreciated in those circles containing higher concentrations of Y_f. Differently, the negative control and the circles containing X_f exhibited the same light green color coming from the uncontrolled residual oxidation of ABTS, Figure 4A.

Mixtures of Y_f and X_f were also investigated in the 2 by 6 array of wax-printed circles. The same green color intensity than samples containing only Y_f were obtained, indicating that the presence of the X_f does not inhibit the formation of the Y_f - DNAzyme (supporting information Figure SI-5).

The quantification of the DNAzyme reaction was done analyzing the color intensity of the circles (green color intensity) by just taking pictures of the array with a mobile phone color camera. The whole area of the reaction circle was selected, without differentiating the presence or absence of the coffee ring. Figure 4 B shows the plot of the color intensity *versus* Y_f and X_f concertation. As qualitatively observed by eye before, the green color intensity increased with the concentration of Y_f, while it remained constant for the assay containing X_f. There was a significant difference in the signal obtained with and without Y_f in all the cases, indicating that this assay could be used in paper for quantification of specific fragments of ssDNA. Y_f values were fitted to a 4PL curve. A LoD of 45.7 ng and a LoQ of 172 ng of Y_f were calculated using eq. 1 and eq. 2, respectively, obtaining a dynamic range of 172 - 1200 ng, which is much lower than in solution. Therefore, transfer the reaction to a paper support greatly improved the LoD, reducing 14 times the minimum amount of Y_f that can be detected. This improvement can be attributed to the higher concentrations of reagents with respect to the detection zone available in the paper substrate. Moreover, after quantification it can be concluded that at least 143 ng of Y amelogenin fragment could be detected by naked eye.

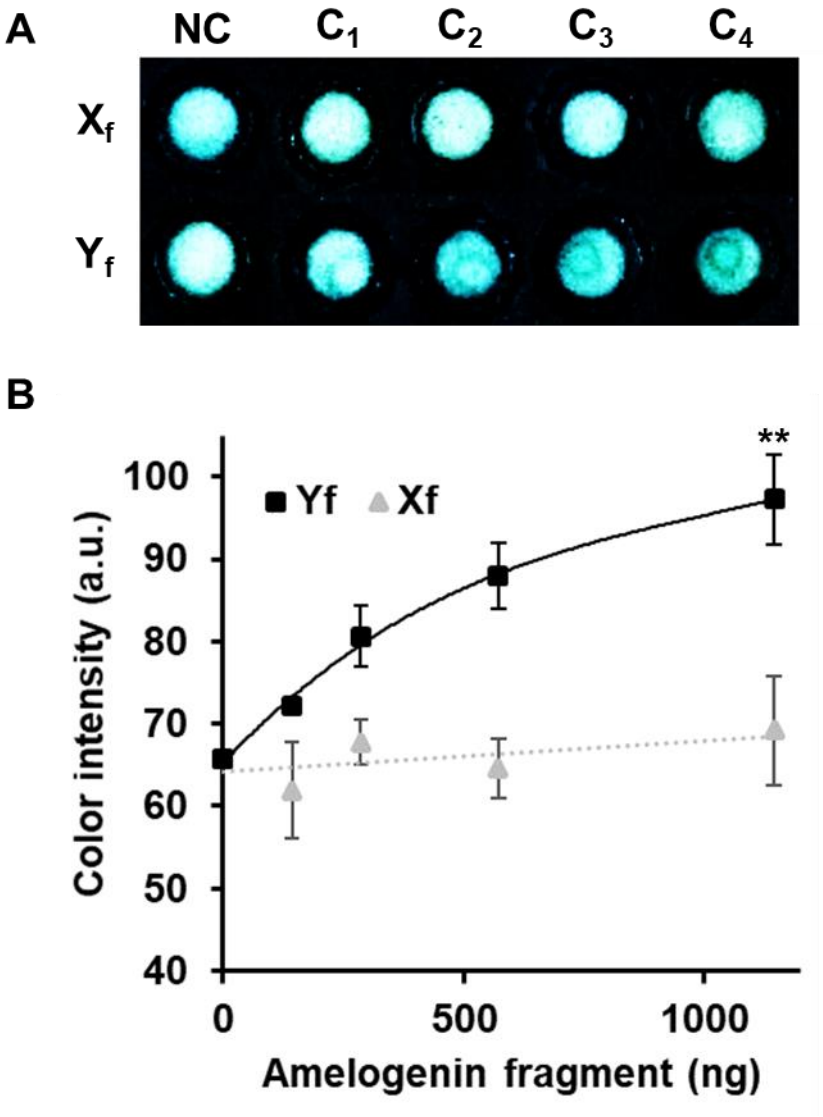

Figure 4. Pictures and graphical representation of Y_f detection on Whatman filter paper 1. A) Picture of the 2 by 6 array printed in paper after 5 min DNAzyme reaction. C_1, C_2, C_3 and C_4 refers to the different concentrations of Y_f or X_f solutions added to the paper substrate (25 (143 ng), 50 (286 ng), 100 (572 ng) and 200 (1144 ng) μM, respectively) and NC is the negative control. B) Graphical representation of the color intensity obtained from the picture taken by the mobile camera 5 min after reaction. Fitting curve of Y_f was done through the equation $y = 97-(31.44/(1+(x/51.7)^{1.23}))$, $R^2 = 0.9917$. Error bars mean ± SD (n = 3 samples per experimental condition). Statistical significance; paired two-tailed t-test (** $p < 0.01$).

In order to fabricate a paper-based device useful in real applications, it is desirable that the reagents are stable for long period of time embedded in the device, preferably in a controlled environment. Therefore, the stability of the reagents (SP1, SP2, hemin and ABTS) forming the assay was tested by storing them at 4 ºC from 24 h to 48 h and 7 days, see Supporting Information Figure SI-6. For the three times investigated, the "coffee ring" was clearly visible in all samples containing Y_f while the green color was imperceptible with the naked eye for the X_f samples, See Figure SI-6A. Pictures of the wax-circles were taken and analyzed, see Figure SI-6B, allowing for the quantification of the ABTS oxidation process. The values obtained for each experiment are comparable within the error (n = 3 experiments), indicating that the reagents are well preserved in the paper substrate maintaining their chemical properties at least for one week, under refrigeration.

3.3 DNA samples analysis on a paper-based device.

A final test was performed to evaluate the applicability of Y_f detection on a paper –based device for the simultaneous analysis of Y_f and X_f samples. As a proof of concept a microfluidic paper-based device was designed and wax-printed. It has a channel with two analysis zones, one at each edge of the channel and an inlet for reagent loading in the middle of the channel (Figure 5). The Y_f - DNAzyme cocktail was pre-loaded in both analysis zones at kept for 24 h at 4 ºC (dark conditions). Then, two samples containing 570 ng of Y_f or X_f were loaded in each analysis zone and let dry for 30 min. Finally, 12 μL of H_2O_2 (1.32 mM) were pipetted in the inlet of the device. The H_2O_2 flowed equally in both detections reaching the analysis zones in less than 10 s. The ABTS oxidation reaction took place and a clear green coffee ring appeared only in the analysis zone containing Y_f after 5 min (supporting information video in SI-7) while the intensity of the green color in the analysis zone containing the X_f sample was very low.

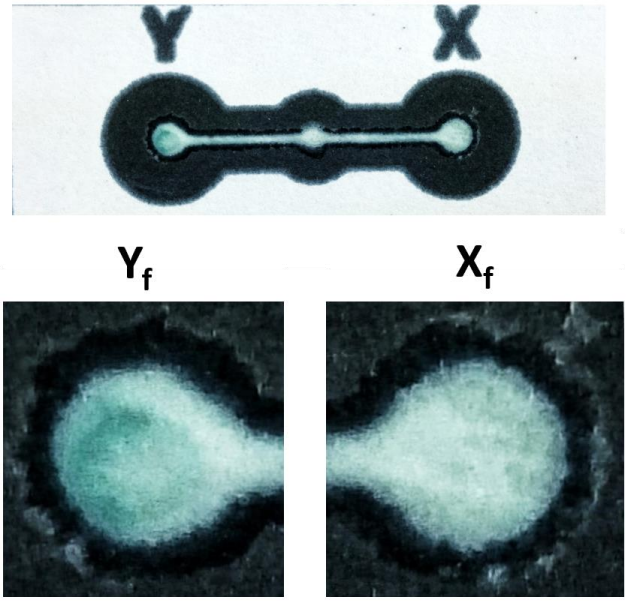

Figure 5. Paper-based device for Y_f detection. Pictures were taken 5 min after the H_2O_2 solution reached the analysis zones. Detection circles correspond to sample loading of Y_f (left) and X_f (right). Increased green intensity relates to increasing presence of $ABTS^{+\cdot}$.

4. CONCLUSIONS

Rapid tests for DNA detection have application in forensic practice, biomedical diagnostics, and environmental monitoring among others. Current ways to analyze DNA sequences often require long procedures at centralized labs, and there is a need to developed technologies that enable in-field and rapid sample analysis and detection of the DNA of interest.

An optimized methodology for easy and in-field ssDNA detection in a paper-based device was presented. By using a DNAzyme specific to the desired DNA sequence, a label-free, easy to use and colorimetric based detection method for ssDNA was developed. This reaction was optimized in solution in order to obtain a distinguishable specific and positive signal in a short time with a LOD of 655 ng.

The assay developed in solution was then transferred to a paper support and to a paper-based device. Two types of paper were tested, Whatman filter paper 1 and nitrocellulose membrane, both highly used in paper microfluidics and/or DNA research [6–8]. It was found out that Whatman filter paper 1 was more adequate for the DNAzyme reaction and thus was chosen as the desired substrate. The DNAzyme reaction on paper enabled the unequivocal colorimetric identification of the Y_f ssDNA fragment by qualitative detection, with the naked eye, and quantitative determination by image analysis. At least 143 ng of Y amelogenin fragment could be detected by naked eye, while 45.7 ng could be detected by the quantification of the color intensity values from a picture of the paper substrate. The transfer of the reaction to a paper support significantly reduced the LoD, improving the sensitivity of the reaction.

Multisampling analysis on a paper-based device pre-loaded with the DNAzyme cocktail was carried out. Although the configuration of the device is not optimized, two analytes, Y_f and X_f, were analysed. The analysis of different genes from a single individual would need of further validation and optimization of the process, including device flow control and prevention of cross-contamination of samples.Furthermore, the devices were stored for up to 7 days at 4 ºC before use, indicating that they could be stored and used at the point of care

This work constitutes the first steps into what could become an in-field paper-based device for several applications. In the case of fast typifying of human samples for forensic analysis it should be noted that human samples are more complex than the proposed 74 bases synthetic sequence, usually presenting the genome in a double strand structure with high packing, safeguarded inside the cell's nucleus. Also, while the detection limits achieved are optimal for our synthetic sequence, the total genetic material found per human cell can be very low (between 3 and 6 picograms per cell) and the amount of cells that can be found in human samples can vary significantly, depending on the sample origins[41], being this scenario a limitation of this technology in its current configuration. Therefore, for the incorporation of real human samples, research on the integration and the optimization of

extraction methods (Chelex 100 [42]) and DNA denaturalization methods (chemical denaturalization [43]) inside the paper supports would be necessary. Conventional techniques for human genoma analysis, such as qPCR, only require nanograms of whole genomes to produce data with high specificity. Our proposed methodology would extremely benefits from complementary amplification methods of the genetic material, such as isothermal amplification, that can be incorporated in the future to an in-field device for the successful detection of human DNA[44,45]. The technology presented in this manuscript could also be applied in the field of biomedicine for the detection and diagnosis of bacterial and viral infections, which present shorter genomes and more variety of DNA structures, including ssDNA[46,47].

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5. REFERENCES

(1) Garai-Ibabe, G.; Möller, M.; Saa, L.; Grinyte, R.; Pavlov, V. Peroxidase-Mimicking DNAzyme Modulated Growth of CdS Nanocrystalline Structures in Situ through Redox Reaction: Application to Development of Genosensors and Aptasensors. *Anal. Chem.* **2014**, *86* (20), 10059–10064. https://doi.org/10.1021/ac502360y.

(2) Abel, G. Current Status and Future Prospects of Point-of-Care Testing around the Globe. *Expert Rev. Mol. Diagn.* **2015**, *15* (7), 853–855.

https://doi.org/10.1586/14737159.2015.1060126.

(3) Claret, P. G.; Bobbia, X.; Roger, C.; Sebbane, M.; De La Coussaye, J. E. Review of Point-of-Care Testing and Biomarkers of Cardiovascular Diseases in Emergency and Prehospital Medicine. *Acta Cardiol.* **2015**. https://doi.org/10.2143/AC.70.5.3110510.

(4) Ren, K.; Chen, Y.; Wu, H. New Materials for Microfluidics in Biology. *Curr. Opin. Biotechnol.* **2014**, *25*, 78–85. https://doi.org/10.1016/j.copbio.2013.09.004.

(5) Akyazi, T.; Saez, J.; Elizalde, J.; Benito-Lopez, F. Fluidic Flow Delay by Ionogel Passive Pumps in Microfluidic Paper-Based Analytical Devices. *Sensors Actuators B Chem.* **2016**, *233*, 402–408. https://doi.org/10.1016/j.snb.2016.04.116.

(6) Sher, M.; Zhuang, R.; Demirci, U.; Asghar, W. Paper-Based Analytical Devices for Clinical Diagnosis: Recent Advances in the Fabrication Techniques and Sensing Mechanisms. *Expert Review of Molecular Diagnostics*. 2017. https://doi.org/10.1080/14737159.2017.1285228.

(7) Zou, Y.; Mason, M. G.; Wang, Y.; Wee, E.; Turni, C.; Blackall, P. J.; Trau, M.; Botella, J. R. Nucleic Acid Purification from Plants, Animals and Microbes in under 30 Seconds. *PLoS Biol.* **2017**. https://doi.org/10.1371/journal.pbio.2003916.

(8) Lu, Y.; Shi, W.; Qin, J.; Lin, B. Fabrication and Characterization of Paper-Based Microfluidics Prepared in Nitrocellulose Membrane By Wax Printing. *Anal. Chem.* **2010**, *82* (1), 329–335. https://doi.org/10.1021/ac9020193.

(9) Castro, C.; Rosillo, C.; Tsutsui, H. Characterizing Effects of Humidity and Channel Size on Imbibition in Paper-Based Microfluidic Channels. *Microfluid. Nanofluidics* **2017**. https://doi.org/10.1007/s10404-017-1860-4.

(10) Carrilho, E.; Martinez, A. W.; Whitesides, G. M. Understanding Wax Printing: A Simple Micropatterning Process for Paper-Based Microfluidics. *Anal. Chem.* **2009**.

https://doi.org/10.1021/ac901071p.

(11) Ma, J.; Yan, S.; Miao, C.; Li, L.; Shi, W.; Liu, X.; Luo, Y.; Liu, T.; Lin, B.; Wu, W.; et al. Paper Microfluidics for Cell Analysis. *Advanced Healthcare Materials*. 2019. https://doi.org/10.1002/adhm.201801084.

(12) Shen, L.; Hagen, J. A.; Papautsky, I. Point-of-Care Colorimetric Detection with a Smartphone. *Lab Chip* **2012**, *12* (21), 4240. https://doi.org/10.1039/c2lc40741h.

(13) Katis, I. N.; Holloway, J. A.; Madsen, J.; Faust, S. N.; Garbis, S. D.; Smith, P. J. S.; Voegeli, D.; Bader, D. L.; Eason, R. W.; Sones, C. L. Paper-Based Colorimetric Enzyme Linked Immunosorbent Assay Fabricated by Laser Induced Forward Transfer. *Biomicrofluidics* **2014**, *8* (3), 036502. https://doi.org/10.1063/1.4878696.

(14) Qiu, X.; Mauk, M. G. An Integrated, Cellulose Membrane-Based PCR Chamber. *Microsyst. Technol.* **2015**. https://doi.org/10.1007/s00542-014-2123-x.

(15) Seok, Y.; Joung, H.-A.; Byun, J.-Y.; Jeon, H.-S.; Shin, S. J.; Kim, S.; Shin, Y.-B.; Han, H. S.; Kim, M.-G. A Paper-Based Device for Performing Loop-Mediated Isothermal Amplification with Real-Time Simultaneous Detection of Multiple DNA Targets. *Theranostics* **2017**, *7* (8), 2220–2230. https://doi.org/10.7150/thno.18675.

(16) Teengam, P.; Siangproh, W.; Tuantranont, A.; Vilaivan, T.; Chailapakul, O.; Henry, C. S. Multiplex Paper-Based Colorimetric DNA Sensor Using Pyrrolidinyl Peptide Nucleic Acid-Induced AgNPs Aggregation for Detecting MERS-CoV, MTB, and HPV Oligonucleotides. *Anal. Chem.* **2017**. https://doi.org/10.1021/acs.analchem.7b00255.

(17) Platella, C.; Riccardi, C.; Montesarchio, D.; Roviello, G. N.; Musumeci, D. G-Quadruplex-Based Aptamers against Protein Targets in Therapy and Diagnostics. *Biochim. Biophys. Acta - Gen. Subj.* **2017**, *1861* (5), 1429–1447. https://doi.org/10.1016/j.bbagen.2016.11.027.

(18) Zhang, S.; Wu, Y.; Zhang, W. G-Quadruplex Structures and Their Interaction Diversity with Ligands. *ChemMedChem* **2014**, *9* (5), 899–911. https://doi.org/10.1002/cmdc.201300566.

(19) Navarro, E.; Serrano-Heras, G.; Castaño, M. J.; Solera, J. Real-Time PCR Detection Chemistry. *Clin. Chim. Acta* **2015**, *439*, 231–250. https://doi.org/10.1016/j.cca.2014.10.017.

(20) Roembke, B. T.; Nakayama, S.; Sintim, H. O. Nucleic Acid Detection Using G-Quadruplex Amplification Methodologies. *Methods* **2013**, *64* (3), 185–198. https://doi.org/10.1016/j.ymeth.2013.10.003.

(21) Ruttkay-Nedecky, B.; Kudr, J.; Nejdl, L.; Maskova, D.; Kizek, R.; Adam, V. G-Quadruplexes as Sensing Probes. *Molecules* **2013**, *18* (12), 14760–14779. https://doi.org/10.3390/molecules181214760.

(22) Silverman, S. K. Catalytic DNA: Scope, Applications, and Biochemistry of Deoxyribozymes. *Trends Biochem. Sci.* **2016**, *41* (7), 595–609. https://doi.org/10.1016/j.tibs.2016.04.010.

(23) Zhou, W.; Saran, R.; Liu, J. Metal Sensing by DNA. *Chem. Rev.* **2017**, *117* (12), 8272–8325. https://doi.org/10.1021/acs.chemrev.7b00063.

(24) Xiao, Y.; Pavlov, V.; Niazov, T.; Dishon, A.; Kotler, M.; Willner, I. Catalytic Beacons for the Detection of DNA and Telomerase Activity. *J. Am. Chem. Soc.* **2004**, *126* (24), 7430–7431. https://doi.org/10.1021/ja031875r.

(25) Fu, R.; Li, T.; Lee, S. S.; Park, H. G. DNAzyme Molecular Beacon Probes for Detection of Nucleic Acids. *Anal. Chem.* **2011**.

(26) Li, W.; Li, Y.; Liu, Z.; Lin, B.; Yi, H.; Xu, F.; Nie, Z.; Yao, S. Insight into G-Quadruplex-Hemin DNAzyme/RNAzyme: Adjacent Adenine as the Intramolecular Species for Remarkable Enhancement of Enzymatic Activity. *Nucleic Acids Res.* **2016**, *44* (15), 7373–

455 7384. https://doi.org/10.1093/nar/gkw634.

456 (27) Zhang, Y.; Fan, J.; Nie, J.; Le, S.; Zhu, W.; Gao, D.; Yang, J.; Zhang, S.; Li, J. Timing
457 Readout in Paper Device for Quantitative Point-of-Use Hemin/G-Quadruplex DNAzyme-
458 Based Bioassays. *Biosens. Bioelectron.* **2015**, *73*, 13–18.
459 https://doi.org/10.1016/j.bios.2015.04.081.

460 (28) Ali, M. M.; Brown, C. L.; Jahanshahi-Anbuhi, S.; Kannan, B.; Li, Y.; Filipe, C. D. M.;
461 Brennan, J. D. A Printed Multicomponent Paper Sensor for Bacterial Detection. *Sci. Rep.*
462 **2017**, *7* (1), 12335. https://doi.org/10.1038/s41598-017-12549-3.

463 (29) Kehoe, E.; Penn, R. L. Introducing Colorimetric Analysis with Camera Phones and Digital
464 Cameras: An Activity for High School or General Chemistry. *J. Chem. Educ.* **2013**, *90* (9),
465 1191–1195. https://doi.org/10.1021/ed300567p.

466 (30) von Wurmb-Schwark, N.; Bosinski, H.; Ritz-Timme, S. What Do the X and Y Chromosomes
467 Tell Us about Sex and Gender in Forensic Case Analysis? *J. Forensic Leg. Med.* **2007**, *14*
468 (1), 27–30. https://doi.org/10.1016/j.jcfm.2005.09.003.

469 (31) Iwase, M.; Satta, Y.; Takahata, N. Sex-Chromosomal Differentiation and Amelogenin Genes
470 in Mammals. *Mol. Biol. Evol.* **2001**, *18* (8), 1601–1603.
471 https://doi.org/10.1093/oxfordjournals.molbev.a003948.

472 (32) Tozzo, P.; Giuliodori, A.; Corato, S.; Ponzano, E.; Rodriguez, D.; Caenazzo, L. Deletion of
473 Amelogenin Y-Locus in Forensics: Literature Revision and Description of a Novel Method
474 for Sex Confirmation. *J. Forensic Leg. Med.* **2013**, *20* (5), 387–391.
475 https://doi.org/10.1016/j.jflm.2013.03.012.

476 (33) Dutta, S.; Nath, P. A Fully Automated Colorimetric Sensing Device Using Smartphone for
477 Biomolecular Quantification. In *Optics and Biophotonics in Low-Resource Settings III*; 2017.

https://doi.org/10.1117/12.2251101.

(34) Findlay, J. W. A.; Dillard, R. F. Appropriate Calibration Curve Fitting in Ligand Binding Assays. *AAPS J.* **2007**. https://doi.org/10.1208/aapsj0902029.

(35) Cui, X.; Li, R.; Liu, X.; Wang, J.; Leng, X.; Song, X.; Pei, Q.; Wang, Y.; Liu, S.; Huang, J. Low-Background and Visual Detection of Antibiotic Based on Target-Activated Colorimetric Split Peroxidase DNAzyme Coupled with Dual Nicking Enzyme Signal Amplification. *Anal. Chim. Acta* **2018**, *997*, 1–8. https://doi.org/10.1016/j.aca.2017.10.009.

(36) Zhang, Y.; Li, B. Reducing Background Signal of G-Quadruplex-Hemin DNAzyme Sensing Platform by Single-Walled Carbon Nanotubes. *Biosens. Bioelectron.* **2011**. https://doi.org/10.1016/j.bios.2011.06.030.

(37) Kadnikova, E. N.; Kostić, N. M. Oxidation of ABTS by Hydrogen Peroxide Catalyzed by Horseradish Peroxidase Encapsulated into Sol-Gel Glass. Effects of Glass Matrix on Reactivity. *J. Mol. Catal. B Enzym.* **2002**. https://doi.org/10.1016/S1381-1177(02)00057-7.

(38) Konan, K. V.; Le Tien, C.; Mateescu, M. A. Electrolysis-Induced Fast Activation of the ABTS Reagent for an Antioxidant Capacity Assay. *Anal. Methods* **2016**. https://doi.org/10.1039/c6ay01088a.

(39) Venkatasubramanian, L.; Maruthamuthu, P. Kinetics and Mechanism of Formation and Decay of 2,2′-azinobis-(3-ethylbenzothiazole-6-sulphonate) Radical Cation in Aqueous Solution by Inorganic Peroxides. *Int. J. Chem. Kinet.* **1989**. https://doi.org/10.1002/kin.550210604.

(40) Ghosh, R.; Vaishampayan, V.; Mahapatra, A.; Malhotra, R.; Balasubramanian, S.; Kapoor, A. Enhancement of Limit of Detection by Inducing Coffee-Ring Effect in Water Quality Monitoring Microfluidic Paper-Based Devices. *Desalin. Water Treat.* **2019**.

https://doi.org/10.5004/dwt.2019.23715.

(41) Rethmeyer, J. A.; Tan, X.; Manzardo, A.; Schroeder, S. R.; Butler, M. G. Comparison of Biological Specimens and DNA Collection Methods for PCR Amplification and Microarray Analysis. *Clinical Chemistry and Laboratory Medicine*. 2013. https://doi.org/10.1515/cclm-2012-0429.

(42) Walsh, P. S.; Metzger, D. A.; Higushi, R. Chelex 100 as a Medium for Simple Extraction of DNA for PCR-Based Typing from Forensic Material. BioTechniques 10(4): 506-13 (April 1991). *Biotechniques* **2013**.

(43) Wang, X.; Lim, H. J.; Son, A. Characterization of Denaturation and Renaturation of DNA for DNA Hybridization. *Environ. Health Toxicol.* **2014**, *29*, e2014007. https://doi.org/10.5620/eht.2014.29.e2014007.

(44) Reboud, J.; Xu, G.; Garrett, A.; Adriko, M.; Yang, Z.; Tukahebwa, E. M.; Rowell, C.; Cooper, J. M. Paper-Based Microfluidics for DNA Diagnostics of Malaria in Low Resource Underserved Rural Communities. *Proc. Natl. Acad. Sci. U. S. A.* **2019**. https://doi.org/10.1073/pnas.1812296116.

(45) Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. Loop-Mediated Isothermal Amplification of DNA. *Nucleic Acids Res.* **2000**.

(46) Krupovic, M.; Forterre, P. Single-Stranded DNA Viruses Employ a Variety of Mechanisms for Integration into Host Genomes. *Ann. N. Y. Acad. Sci.* **2015**. https://doi.org/10.1111/nyas.12675.

(47) Labonté, J. M.; Suttle, C. A. Previously Unknown and Highly Divergent SsDNA Viruses Populate the Oceans. *ISME J.* **2013**. https://doi.org/10.1038/ismej.2013.110.