analytical chemistry

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# Development of an Innovative Quantification Assay Based on Aptamer Sandwich and Isothermal Dumbbell Exponential Amplification

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Cite This: *Anal. Chem.* 2022, 94, 3376−3385 Read Online

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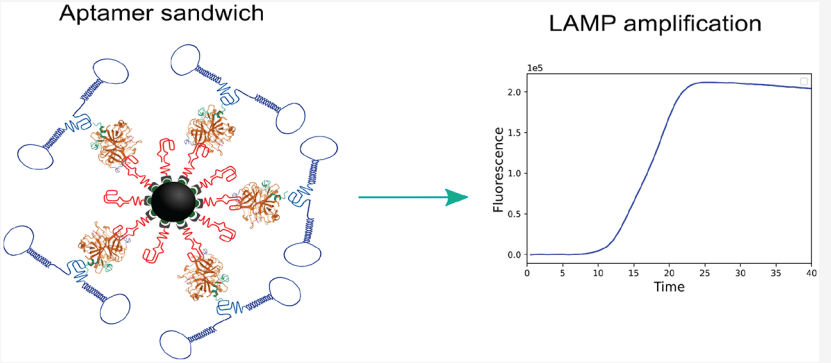


**ABSTRACT:** Detecting blood biomarkers such as proteins with high sensitivity and specificity is of the utmost importance for early and reliable disease diagnosis. As molecular probes, aptamers are raising increasing interest for biosensor applications as an alternative to antibodies, which are used in classical enzyme-linked immuno-sorbent assays (ELISA). We have developed a sensitive and antibody-free molecular quantification assay that combines the specificity of aptamers and the sensitivity of the loop-mediated isothermal amplification (LAMP). For the proof-of-concept, we consider two types of biomarkers: (i) a model of oligonucleotide mimicking nucleic acid targets and (ii) the thrombin involved in the complex coagulation cascade as a model protein for which two relevant aptamers form a stable sandwich. The assay protocol is based on a few successive steps, similar to sandwich ELISA. First, aptamer-coated magnetic beads are added to the sample to specifically capture the targets. Then, the sandwich complex is formed by adding the second aptamer. This secondary aptamer is integrated in a larger oligonucleotide dumbbell sequence designed for LAMP detection using only two primers. After a proper rinsing step, the isothermal dumbbell exponential amplification is performed to detect and quantify a low amount of targets (limit of detection ∼ 1 pM for the oligonucleotide and ∼100 pM for thrombin). This study demonstrates that our innovative aptamero-LAMP assay could be relevant for the detection of different types of biomarkers and their quantification at physiological levels. This may also pave the way for antibody-free molecular assays.

## INTRODUCTION

The quantification of a target present at very low concentrations in a complex sample currently remains a challenge. Different techniques have been developed in the past, and the most commonly used is the enzyme-linked immuno-sorbent assay (ELISA) which was developed in the early 1970s.[1] ELISA is based on a sandwich of the target formed by two antibodies followed by an enzymatic amplification and is widely used for all types of assays, either direct or indirect, as well as for various targets (proteins, biomarkers, or even viruses and cells).[2−4] However, the ELISA techniques are sometimes limited or even ineffective when the target to be detected is present at very low concentrations in the sample.[4−6] In order to solve this problem, more efficient amplification strategies such as DNA probe amplification may be relevant. Indeed, while enzymatic amplification is linear in time, exponential oligonucleotide amplifications are possible[7] like in the polymerase chain reaction (PCR).[8] Such amplification methods have been developed as alternative detection methods to ELISA. Particular examples of these

**Received:** December 22, 2021
**Accepted:** January 31, 2022
**Published:** February 10, 2022





https://doi.org/10.1021/acs.analchem.1c05532
*Anal. Chem.* 2022, 94, 3376−3385

Table 1. Oligonucleotide Sequences

| name | length (bases) | sequence (5′−3′) | specific feature |
|---|---|---|---|
| Thr1 | 15 | GGTTGGTGTGGTTGG | thrombin aptamer 1 |
| Thr2 | 29 | AGTCCGTGGTAGGGCAGGTT GGGGTGACT | thrombin aptamer 2 |
| Zipc | 24 | CGCATACCAGGTCGCATACCGGTC | capture probe |
| ZipThr1c | 39 | CCAACCACACCAACCGACCGG TATGCGACCTGGTATGCG | oligonucleotide target |
| B1 | 25 | GGGGGAAAGATATAACTCAGAGATG | |
| B2 | 18 | GAAGGAGGGTCAGTGAGG | |
| F1 | 21 | ATAAACCGCGTCTTGGATCCG | |
| F2 | 24 | CGTGCAGTACGCCAACCTTTCTCA | |
| FIP | 45 | F1c−F2 | forward inner primer |
| BIP | 43 | B1c−B2 | backward inner primer |
| D1 | 134 | F1c−F2−F1−B1c−B2c−B1 | generic dumbbell |
| D2 | 152 | F1c−F2−F1−Thr1−B1c−B2c−B1 | central Thr1 dumbbell |
| D3 | 152 | F1c−F2−Thr1−F1−B1c−B2c−B1 | F loop Thr1 dumbbell |
| D4 | 152 | F1c−F2−F1−B1c−Thr1−B2c−B1 | B loop Thr1 dumbbell |

methods are immuno-PCR and aptamero-PCR. In immuno-PCR,[9−13] the enzymatic amplification of the ELISA technique is replaced by DNA amplification. In this case, the secondary antibody is coupled to a nucleic acid sequence, which is then exponentially amplified by PCR. This powerful immuno-detection method combines the specificity and versatility of an ELISA with the exponential signal amplification enabled by PCR. Immuno-PCR is highly specific and quantitative[9−13] but still requires the presence of two target-specific antibodies and the development of a DNA-conjugated antibody, which makes this method complicated and expensive.[14] Aptamero-PCR is also based on the principle of PCR amplification, but the secondary antibody is replaced by an oligonucleotide containing an aptamer sequence specific to the target to be detected and primer regions required for the PCR amplification.[15] This secondary probe is thus composed of a single oligonucleotide. Such a detection method is a combination of immuno-PCR and enzyme-linked aptamer(-sorbent) assays (ELASA or ELAA).[16,17] While relatively uncommon, the high sensitivity of the amplification and the use of aptamers as a substitute for antibodies make this method very interesting for the detection of all types of targets, particularly in complex media.[18] Recently, a team has developed a sensitive diagnostic test for the influenza A virus using aptamero-PCR with validation on clinical samples.[19] Nonetheless, both immuno-PCR and aptamero-PCR have the drawbacks of PCR amplification, which are the need for temperature cycles and a high sensitivity to inhibitors that may be present in the samples. Thus, different DNA amplification methods have been developed in recent years to replace PCR.[20] Among them, the loop-mediated isothermal amplification (LAMP), developed in the early 2000s,[21] presents the major advantage of being carried out at a constant temperature, typically around 65 °C. LAMP appears to be a promising alternative to PCR as one of the most specific and sensitive methods of all isothermal DNA amplification techniques[20,22] and less sensitive to inhibitors present in biologial samples.[23,24] Furthermore, the isothermal nature of LAMP results in reduced power consumption and makes it easier to integrate into a portable device.[25,26]

Several teams have worked on the coupling of aptamer probes and isothermal DNA amplification methods,[27] but only a few teams have considered LAMP. Aptamero-LAMP was first theoretically described for the detection of proteins.[28] An aptamer (or antibody) attached to a surface first captures the target. An oligonucleotide containing a second aptamer specific to the target then forms a sandwich. This secondary oligonucleotide is finally amplified by the conventional LAMP method. Soon after, the team of Yuan developed a method where the aptamer used for the recognition of the small molecule target ochratoxin A was used as a primer in the LAMP technique.[29,30] In 2017, Cao et al. succeeded in the detection of the Mucin-1 protein—a biomarker for cancer diagnosis.[31] Magnetic beads coated with streptavidin are incubated with Mucin-1 proteins previously coupled to biotin molecules. As targets are functionalized, this method does not suit for real and complex samples. Two years later, another team developed a microfluidic device enabling the detection of the H1N1 virus responsible for influenza A.[22] The sample containing the viruses is incubated with magnetic beads functionalized by specific aptamers of the virus. After rinsing, the viral RNAs are released following the lysis of the viruses and then brought into contact with the reagents necessary for RT-LAMP amplification. In this case, the method does not rely on the aptamer probe amplification but on the viral RNA amplification, which cannot be applied to protein detection. More recently, an example of protein detection by an aptamer followed by an original but complex LAMP amplification was described.[32] The method is based on the recognition of thrombin by two aptameric sequences. However, many steps were required between the recognition of the target by the aptamers and the start of amplification. Furthermore, the aptamer probes were not directly amplified, leading to the use of several enzymes.

The thrombin protein is the perfect model for a proof-of-concept of aptamero-LAMP since two aptamers have been described to bind two different epitopes of the protein and to form a stable sandwich.[33] On the basis of the couple of thrombin aptamers, we developed an aptamero-LAMP assay with a simplified DNA amplification based on a dumbbell oligonucleotide structure requiring only two primers among the four generally needed for LAMP. Such dumbbell amplification by two primers has already been described in recent studies for miRNA detection.[34−36] In our case, we introduced an aptamer sequence at various positions in the dumbbell structure to analyze its impact on the amplification



https://doi.org/10.1021/acs.analchem.1c05532
Anal. Chem. 2022, 94, 3376−3385

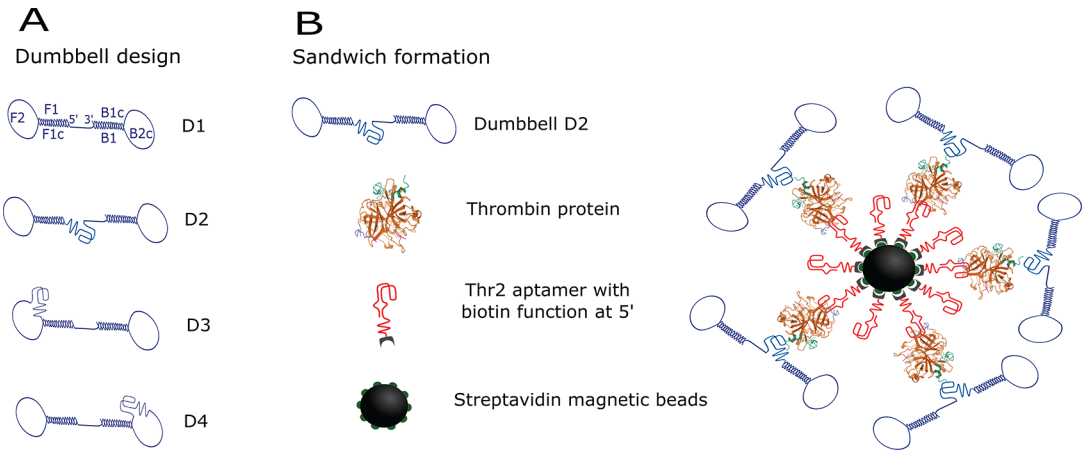


**Figure 1.** (A) Scheme of the different dumbbell designs. D1 is the minimal dumbbell construct and does not contain any aptamer sequence. D2, D3, and D4 present the aptamer in various positions. (B) Scheme of the double aptamer sandwich formation for the capture of thrombin prior to the isothermal dumbbell exponential amplification. Magnetic beads functionalized with Thr2 aptamer were used to capture the target, thrombin protein, and the dumbbell structure incorporating the Thr1 aptamer was used as a recognition probe. After proper rinsing of the nonreacted dumbbells, the remaining ones were then put in contact with a LAMP reaction mix and warmed at 65 °C for 60 min for an exponential isothermal amplification and further quantification.

and recognition of the protein. We validated the amplification of these different dumbbells in less than 30 min and the quantitative detection of thrombin protein with a limit-of-detection of 100 pM and a quantification range of 3 orders of magnitude.

## MATERIALS AND METHODS

**Reagents and Chemicals.** Streptavidin magnetic beads of 1 $\mu$m size (Dynabeads MyOne Streptavidin T1) were purchased from Fischer Scientific (France) and used with a DynaMag Magnet. Smaller particles improve the specific surface accessible for binding while rendering their capture by a standard magnet more difficult. Thus, micrometer-sized magnetic beads are a good compromise. Human $\alpha$-thrombin and $\gamma$-thrombin were purchased from Cryoprep (France). Bovine serum albumine (BSA) was purchased from Sigma-Aldrich (France).

Thrombin was captured in a sandwich format with two aptamers previously described in the literature[37] named Thr1 (5′-GGT TGG TGT GGT TGG-3′)[38] and Thr2 (5′-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′).[39] Thr2 was modified at the 5′ end with a biotin for grafting onto the streptavidin magnetic beads and a spacer of 10 thymines, allowing a better flexibility and accessibility of the aptamer. The Thr1 sequence was incorporated in a larger oligonucleotide dumbbell sequence to enable its exponential amplification. The dissociation constants $K_D$ for Thr1 and Thr2 were determined in the nanomolar range (respectively 5 and 6 nM).[33] The affinity of the Thr2 aptamer was shown to be slightly affected by the grafting onto a surface whatever its grafting density.[40] Furthermore, the kinetics and stability of the Thr1−thrombin−Thr2 sandwich formation was experimentally assessed previously.[33] The dumbbell sequences used in this study are described in the next section. All of the oligonucleotides were purchased from Eurogentec (France) and are presented in Table 1.

The buffer used to wash the magnetic beads (WB) was composed of 10 mM Tris-HCl, 1 mM EDTA, and 2 mM NaCl, at pH 7.4. The aptamer buffer (AB) used for thrombin−aptamer interactions was inspired by the selection buffer of the aptamers:[33,38,39] 20 mM Tris-HCl, 120 mM NaCl, 1 mM $MgCl_2$, and 10 mM KCl, at pH 7.4. The hybridization buffer (HB) used to favor DNA hybridization was composed of PBS 1×, 1.074 M NaCl, 0.3% Tween 20, 20 $\mu$g/mL of single stranded DNA from salmon testes, and 4% blocking agent Denhart. The rinsing solution (RS) was composed of PBS 1×, 1 M NaCl, and 0.3% Tween 20. All components of the buffers were purchased from Sigma-Aldrich (France).

**Dumbbell Design Method.** The aptamero-LAMP developed in this study relied on a dumbbell oligonucleotide that recognizes the target of interest and can be amplified by an isothermal exponential amplification using only two primers. Such an amplification method based on two primers have already been described and used by several groups for other applications.[34−36] In our aptamero-LAMP, an isothermal dumbbell exponential amplification was performed by removing the first steps of the traditional LAMP technique. Since the oligonucleotide probes are already in a dumbbell structure ready for the cycling amplification, the LAMP primers generally called B3 and F3 necessary for the dumbbell formation are here unnecessary.[21]

The dumbbell probe is a single stranded oligonucleotide with a double stem loop structure. The generic structure D1 presents the following sequence 5′-F1c−F2−F1−B1c−B2c−B1-3′ where Xc stands for the complementary strand of X (see Table 1 for details of the sequences). Only backward inner primer (BIP) and forward inner primer (FIP) are required for the exponential amplification. In several portions of this generic dumbbell, an aptamer sequence specific to the analyte to be detected was introduced without inhibiting the amplification. As we have supposed that the position of the aptamer within the dumbbell influences its conformation and thus its ability to recognize the target, we designed three different aptamer−dumbbells incorporating the aptamer in central position between F1 and B1c for dumbbell D2, in the forward loop (between F2 and F1) for D3 and in the backward loop (between B1c and B2c) for D4. An intermediary sequence with a length up to three bases can be added in between F1 and B1c to give more flexibility. The design of these dumbbells was developed for thrombin sensing. The


https://doi.org/10.1021/acs.analchem.1c05532
Anal. Chem. 2022, 94, 3376−3385

aptamer Thr1 was thus inserted into the dumbbells D2, D3, and D4 in order to be used as a recognition probe in the sandwich format as described in Figure 1. As the position of the aptamer sequence within the dumbbell could influence the efficiency of the isothermal amplification, we have tested its amplification to check its ability to be used in sensing applications.

**Dumbbell Amplification.** Dumbbell amplifications were carried out within a 20 $\mu$L working volume composed of 18 $\mu$L of LAMP mix and 2 $\mu$L of the dumbbell samples at fixed and known concentrations or from the sandwich construct solutions of the aptamero-LAMP assay (see below for description). The LAMP reaction solution was composed of several reagents with final concentrations as follows: FIP at 2.4 $\mu$M and BIP at 2.4 $\mu$M, 1× isothermal amplification buffer (NEB, France), 1 mM $MgSO_4$ (NEB, France), 0.8 M betain (Sigma-Aldrich, France), 1.4 mM of deoxiribonucleotides (dNTPs) solution (Sigma-Aldrich, France), 0.4 U/mL of Bst 2.0 DNA polymerase (NEB, France), and 0.25× of eva green fluorescent DNA (Jena Bioscience, Germany).

Finally, the solution was placed in the QuantStudio 3 Real-Time PCR System (ThermoFischer) and heated at 65 °C for one hour. The fluorescence of the solution was recorded every 30 s. Experimental amplification duplicates called analytical replicates were realized for each samples.

**Single Stranded Oligonucleotide Assay.** To investigate the capacity of the developed method to detect an analyte, an oligonucleotide detection assay based on DNA hybridization was first performed. The affinity of the hybridization of a DNA duplex larger than 15 base pairs is sufficient for the sandwich formation to be complete even at low concentrations of the target. Thus, we expected that the captured dumbbells will be quantitatively equivalent to the amount of oligonucleotides present in the samples.

The detection method is based on a sandwich type detection with a single-stranded DNA target called ZipThr1c, which is complementary to the Thr1 thrombin aptamer sequence present on the dumbbell, and to a Zipc single stranded oligonucleotide immobilized on the magnetic beads. All of the experiments were performed in 1.5 mL LoBind Eppendorf tubes with a working volume of 200 $\mu$L of HB. First, the magnetic beads were coated with an oligonucleotide sequence Zipc by incubating 2 $\mu$M of the oligonucleotide with 5 mg/mL magnetic beads for 10 min at room temperature with shaking. Three washing steps were carried out with WB, and the functionalized magnetic beads were then blocked with the HB containing 1% BSA. These magnetic beads were used to capture the target sequence ZipThr1c at various concentrations from 1 nM to 1 pM for 30 min at 37 °C with shaking. The concentrations of Zipc sequences on the magnetic beads were in excess compared to targets' concentrations. The magnetic beads were washed three times with RS. Then, they were put in contact with the dumbbell D2 at a concentration of 100 pM and incubated for 30 min at 37 °C with shaking. Since, the dumbbells contained the aptamer Thr1, which is complementary to a part of the target sequence ZipThr1c, we expected the hybridization to be complete between targets and dumbbells. The magnetic beads were washed again three times with RS and resuspended in HB. A negative control with no target DNA to form the sandwich was achieved. In parallel, a positive control was performed with a configuration in which the complementary aptamer Thr1c was immobilized on the magnetic beads and was recognized in a direct format by the dumbbell. The dumbbell was added with a concentration range of 1 pM to 1 nM on the Thr1c functionalized magnetic beads. Once again, the concentration of the Thr1c sequences on the magnetic beads was sufficient for a complete capture of the dumbbells in the samples in order to obtain a positive control range as a calibration curve.

**Thrombin Assay with Aptamero-LAMP.** The assay was based on an aptamer sandwich-type detection as presented in Figure 1. The protocols were realized in a microplate well with a 40 $\mu$L working volume. First, the magnetic beads were coated with Thr2 aptamer by incubating 2 $\mu$M oligonucleotide with 5 mg/mL magnetic beads for 10 min at room temperature with shaking. Three washing steps were carried out with WB, and the functionalized magnetic beads were then blocked with the AB containing 1% BSA. These magnetic beads were used to capture the target thrombin protein with concentrations ranging from 1 $\mu$M to 10 pM for 30 min at room temperature with shaking. Unbound proteins were removed using the magnets. A solution containing the dumbbell at a concentration of 100 pM was added to the magnetic beads and incubated for 30 min at room temperature with shaking. Unbound dumbbells were removed with a rinsing step consisting of supernatant removal, and the magnetic beads were resuspended in their initial buffer (AB). In parallel, an amplification of a dumbbell range from 10 nM to 10 pM in a solution (PBS 1×, 5 mM $MgCl_2$) was achieved for each experiment as a positive control. A negative control with no target (Aptamer Buffer) and a specificity control with a nonspecific target (100 nM of $\gamma$-thrombin in AB) were also achieved for each experiment.

**Data Processing for Target Quantification.** After the last step of oligonucleotide or protein assay, 2 $\mu$L of samples (including the magnetic beads) were collected from the wells and deposited into 18 $\mu$L of LAMP mix. To check the mix solution for contamination, a blank assay was performed by using 2 $\mu$L of assay buffer (HB or AB) instead of the sample. Amplification duplicates are systematically achieved.

From the fluorescence data acquired during amplification, the Quant Studio Design and Analysis Software computes time-to-positive ($T_p$) values for each sample, defined as the time required for the fluorescence signal to cross a threshold. Calibration curves were drawn with different analyte or dumbbell concentrations (in log scale) as function of the $T_p$ values. The response linearity was assessed through a linear regression correlation coefficient. In the protocols described above, experimental replicates were systematically achieved. Furthermore, for each sample, amplification replicates were performed.

For the protein detection assay, data were analyzed by taking the time-to-positive value of the 1 $\mu$M thrombin target sample as a reference point. The difference was computed from this point to other thrombin target concentrations ($T_p - T_p([\text{thrombin}] = 1\ \mu\text{M})$). These standardized detection values were drawn along with error bars including both analytical and experimental replicates.

## RESULTS AND DISCUSSION

Before assessing the aptamero-LAMP assay, we analyzed the influence of the Thr1 thrombin aptamer position inside the dumbbell probe on the amplification. Then, we confirmed with a model sandwich assay using oligonucleotide targets the quantitative performances of the amplification. Finally, we


https://doi.org/10.1021/acs.analchem.1c05532
Anal. Chem. 2022, 94, 3376−3385

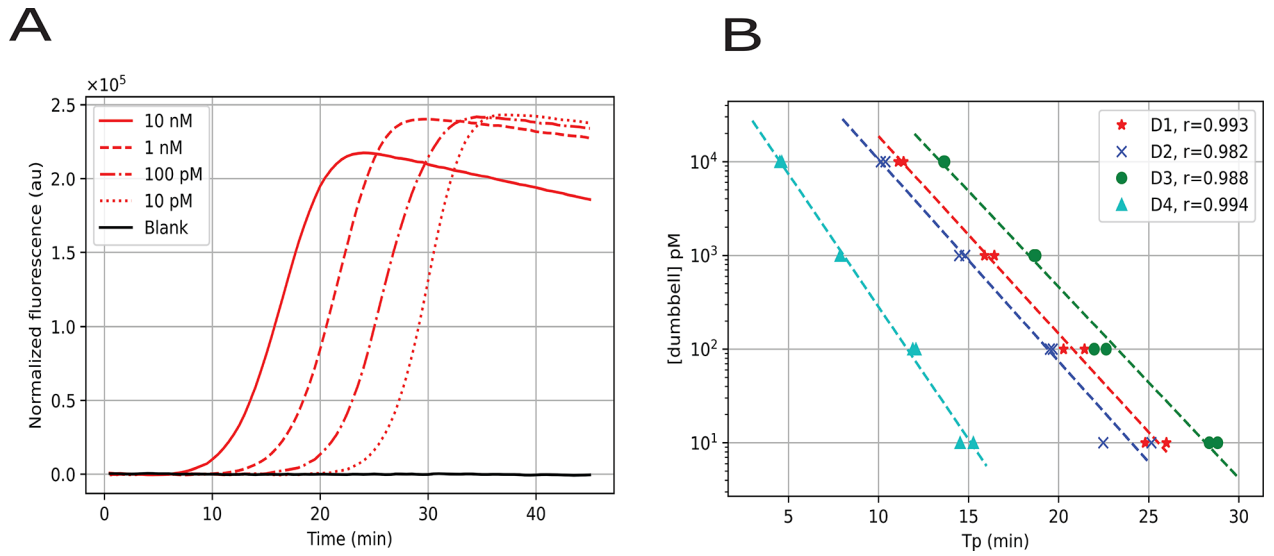


**Figure 2.** Isothermal dumbbell exponential amplification: (A) Normalized fluorescence data over time for dumbbell D1 in a concentration range from 10 pM to 10 nM along with a blank sample without a dumbbell. (B) Logarithm of the dumbbell concentration as function of time-to-positive $T_p$ (in minutes) for all four dumbbells D1, D2, D3, and D4.

analyzed the performance of the aptamero-LAMP assay on thrombin detection.

**Isothermal Dumbbell Exponential Amplification.** We have constructed four dumbbell structures incorporating or not the Thr1 aptamer sequence at different positions. The first step consisted in validating the amplification of the dumbbells and confirming that the aptamer-containing dumbbell sequence is perfectly suitable for amplification. Four dumbbells were tested. D1 contains no aptamer sequence and served as a reference. D2 contains the aptamer sequence in the middle of the dumbbell between both stems, while D3 and D4, respectively, contain the aptamer within the forward and backward loops (see Table 1 and Figure 1). Although the dumbbell sequences are about 150 bases long, they may be synthesized chemically with reasonable yield by a standard manufacturer.

All dumbbell DNA sequences were amplified at various concentrations ranging from 10 pM to 10 nM. In all cases, a clear exponential amplification was observed with larger $T_p$ values for lower concentrations of dumbbells. The example of dumbbell D1 is presented in Figure 2A along with a blank without any dumbbell. The mean value of normalized fluorescence signals of amplification replicates was plotted. The linear correlation between the logarithm of the dumbbell concentration and the $T_p$ values was excellent with $R^2 > 0.98$ for all dumbbells (see Figure 2B). Thus, the quantification of all dumbbells was possible on the 4 orders of magnitude of concentration in less than 30 min. Surprisingly, the reference dumbbell D1 did not present the fastest amplification time as expected due to its shorter length. In fact, the values of $T_p$ for D2 and D3 are similar to the one of the reference dumbbell D1 (less than a few minutes difference at equivalent concentrations). This suggests that the length of the dumbbell (134 bases for D1 and 152 for D2 and D3) does not significantly impact the amplification, neither the incorporation of an aptamer sequence in the middle of the dumbbell nor inside the forward loop. On the contrary, the incorporation of the aptamer sequence inside the backward loop strongly accelerated the exponential amplification especially at low dumbbell concentrations. An amplification in less than 15 min was possible at dumbbell concentrations as low as 10 pM. Furthermore, a decrease of 10 min in detection time of the amplification was observed at 10 pM for D4 compared to D1. Interestingly, the G-quadruplex structure of the Thr1 aptamer did not seem to impact the binding of the BIP and FIP primers or the Bst DNA polymerase activity.

The aptamer sequence position in the dumbbell D4 favorably affected the efficiency of the LAMP amplification. We made the hypothesis that a better affinity for BIP primer binding to the D4 dumbbell may explain this result, by comparison with the other constructions. Dumbbells D2 and D4 are the most optimal in terms of amplification time and reproducibility with a preference for D4. However, the double stem loop conformation of the dumbbell induced strong constraints on the aptamer conformation, especially when the latter was inserted within the loops, which is the case for dumbbells D3 and D4. For the oligonucleotide assay, dumbbells D2 and D4 are both functional, with different performances related to the dumbbell LAMP efficiency, since the assay relies on DNA hybridization. However, for the dumbbell recognition of thrombin protein, the Thr1 aptamer needs to adopt its G-quadruplex conformation,[33,41] which is more constrained with the dumbbell D3 and D4 conformations. We did not obtained thrombin quantification in a double aptamer sandwich format using dumbbell D4. Therefore, for more clarity, we will present in the next section results of an oligonucleotide and protein quantification assay with dumbbell D2, as a generic construction.

**Oligonucleotide Detection and Quantification.** Before analyzing the performance of the aptamero-LAMP assay toward the detection of proteins, we analyzed the detection of a model oligonucleotide both in direct (target directly attached to the magnetic beads) or indirect assays (target free in solution and captured by two probes forming a sandwich). The objective was to check the feasibility of the assay using the sandwich capture and detection with isothermal dumbbell exponential amplification. Due to the strong hybridization between oligonucleotides and their high melting temperature, we expected the reaction to be complete. The direct assay served as a calibration for the indirect one.


https://doi.org/10.1021/acs.analchem.1c05532
Anal. Chem. 2022, 94, 3376−3385

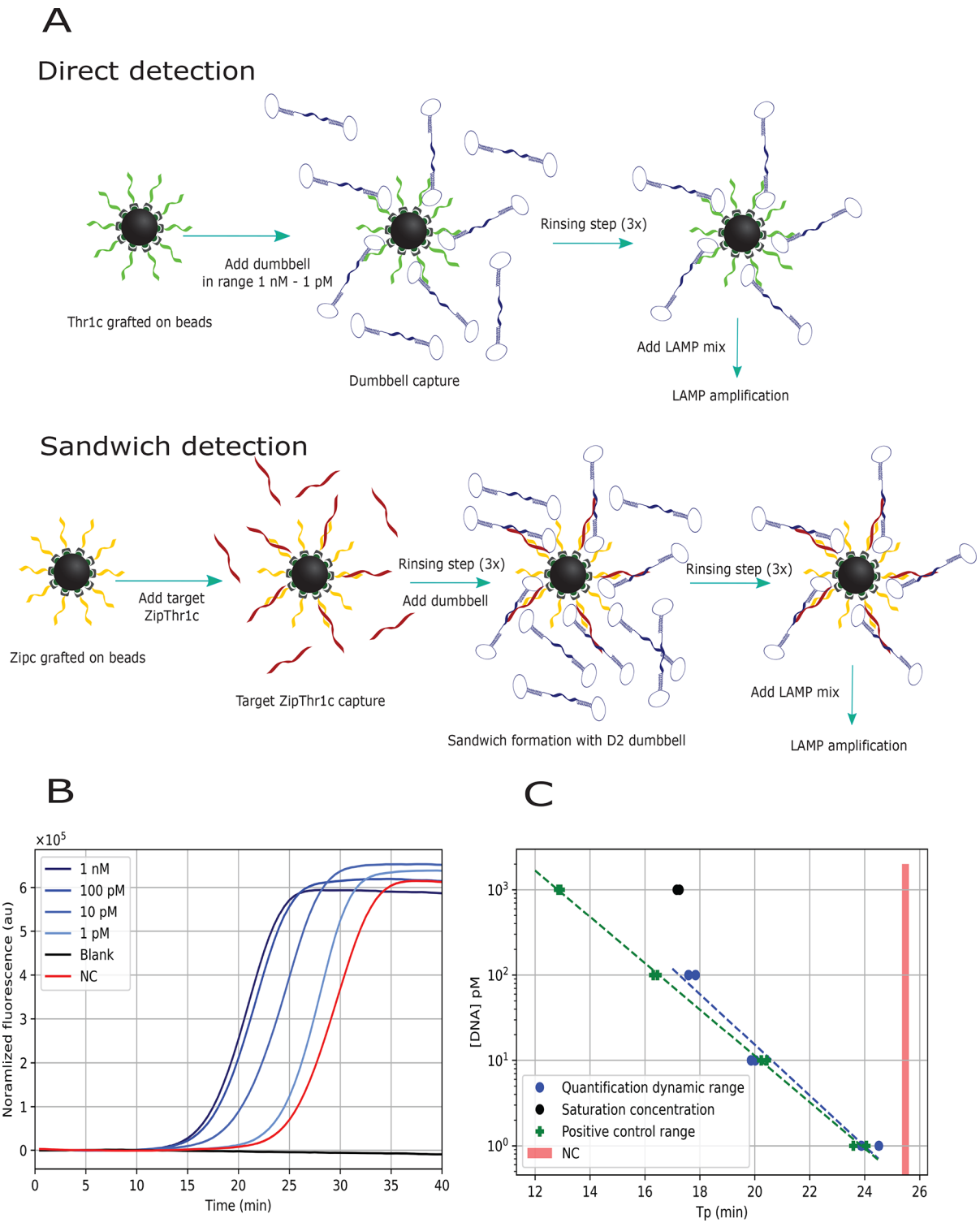


**Figure 3.** Oligonucleotide sandwich capture and isothermal dumbbell exponential amplification. (A) Scheme of the experimental protocol. (B) Mean of normalized fluorescence data ($N = 2$) over time for different concentrations of oligonucleotide targets in solution: 1 nM, 100 pM, 10 pM, and 1 pM. Blank in black. (C) Dynamic range for the indirect detection of oligonucleotides with sandwich capture (blue and black), positive control range of dumbbell D2 concentrations from 1 pM to 1 nM in direct detection assay (green), and negative control (red region). The limit of detection is 1 pM.

In order to get a calibration curve for the indirect detection assay, we first performed four positive control reactions by bringing the dumbbell D2 (from 1 pM to 1 nM) into contact with Thr1c grafted onto magnetic beads. After 30 min of incubation, we expected that all of the dumbbells were captured to targets on the beads since the targets were in excess. Then, after removing the unreacted dumbbells, an amplification was performed. As can be seen in Figure 3C, the $T_p$ presented a similar linear behavior with respect to the initial concentration of dumbbells to the values obtained for the



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Anal. Chem. 2022, 94, 3376−3385

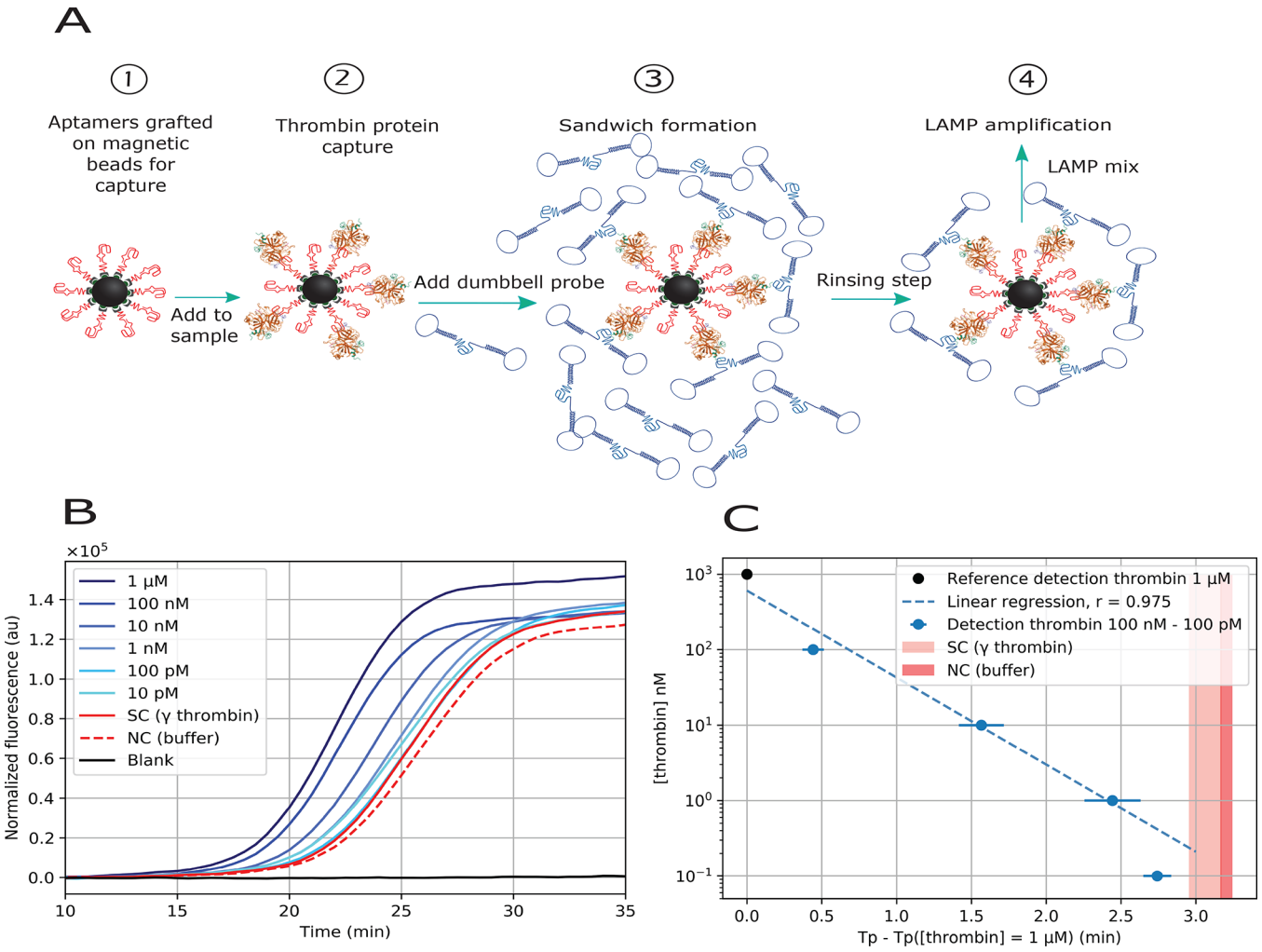


**Figure 4.** Thrombin capture through aptamer sandwich and isothermal dumbbell exponential amplification. (A) Scheme of the protocol. (B) Fluorescence data (arbitrary unit) over time for different concentrations of thrombin targets: 1 $\mu$M, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM. Negative and specificity control are shown in dark and light red, respectively, and blank in black. (C) Dynamic range for experimental replicates standardized with reference to the detection of 1 $\mu$M thrombin (black dot). Three experimental replicates as well as two LAMP analytical replicates were achieved for each sample. Limit of detection was 100 pM, and limit of quantification was around 1 nM. Linear fit within the range 1 $\mu$M to 1 nM showed a satisfactory regression (dashed line, $R^2$ = 0.975).

dumbbell-free experiment in solution (Figure 2B). It suggests that all of the dumbbells have indeed hybridized to the targets grafted onto the magnetic beads.

Next, we performed an indirect assay for the detection and quantification of an oligonucleotide free in solution. The targets ZipThr1c at various concentrations were first hybridized onto magnetic beads functionalized with Zipc for 30 min. Due to the excess of Zipc probes, we expected that all of the targets were captured. After rinsing, the magnetic beads were put into contact with 100 pM of dumbbell D2 for 30 min. We expected that the Thr1 aptamer sequence from the dumbbell D2 hybridized with the targets bound to the magnetic beads. Finally, another rinsing was performed to ensure that nonreacted dumbbells were removed before amplification (Figure 3A). The fluorescence observed over time during the amplification is presented in Figure 3B for a range of target concentrations between 1 nM and 1 pM along with a blank sample. For the lower target concentrations, the fluorescence signal started increasing exponentially at later times, as expected. The negative control without targets increased even later, suggesting that nonspecific interactions of the dumbbells may occur and lead to finite $T_p$ responses. However, the full range of target concentrations presented $T_p$ values shorter than the negative control, suggesting a detection limit lower than 1 pM.

Indeed, the $T_p$ values for various concentrations of targets presented a linear behavior with the logarithm of the target concentration in the range 1−100 pM (blue dots in Figure 3C). More interestingly, those values are comparable to the calibration with the direct assay at similar dumbbell concentrations (green dots in Figure 3C). This confirmed that both Zip−Zipc and Thr1−Thr1c hybridizations were fully completed. On the contrary, at a higher target concentration (1 nM, black dot in Figure 3C), the dumbbell at a concentration of 100 pM was the limiting reagent in the sandwich, and thus the $T_p$ value was comparable to the one observed with 100 pM of targets. This saturation condition sustained a higher limit of quantification of 100 pM. At lower concentrations than 1 pM, the results would be blurred by the nonspecific interactions (red region of the blank experiment in Figure 3C), thus confirming a limit of detection of 1 pM for oligonucleotide targets.



https://doi.org/10.1021/acs.analchem.1c05532
Anal. Chem. 2022, 94, 3376−3385

This proof of concept of an oligonucleotide quantification was a way to validate the double sandwich model followed by isothermal dumbbell exponential amplification. The G-quadruplex folding of the aptamer inside dumbbell D2 did not seem to affect the hybridization of the oligonucleotide. The dynamic range achieved in this case could potentially be optimized further with the use of faster Bst DNA polymerase or with stronger rinsing steps to reduce the nonspecific interactions. Moreover, this proof of concept is a way to validate the functionality of the dumbbell to recognize single stranded oligonucleotides in a sandwich format.

**Thrombin Quantification Assay.** Even though dumbbell D4 presented the fastest amplification of the four designed dumbbells, it was probably not relevant to detecting proteins. Indeed, the aptamer inserted in the backward loop of the dumbbell certainly presented too many constraints, and therefore its G-quadruplex structure may be affected and lead to unfavorable protein recognition. However, the dumbbell D2 used in the sandwich assay should surely present good affinity toward the target protein because of the central position of the Thr1 aptamer and should allow quantification. Therefore, we performed the thrombin capture using a sandwich format with the D2 dumbell (Figure 4A). As may be seen in Figure 4B, for lower thrombin concentrations, the exponential amplification started later, suggesting fewer dumbbells forming a sandwich with thrombin. More interestingly, we observed a strong correlation ($R^2$ = 0.975) between the standardized $T_p$ values and the concentrations of thrombin within the range 1 nM to 1 $\mu$M (see Figure 4C). Thus, a quantification of thrombin concentration is possible within 3 orders of magnitude in the physiological range.[42,43] Furthermore, the negative control with a buffer solution presented significantly different results from the solution containing 100 pM thrombin (see Figure 4C), suggesting a detection limit of 100 pM.

The specificity of the aptamero-LAMP assay was assessed using $\gamma$-thrombin, the proteolyzed derivative of $\alpha$-thrombin. $\gamma$-thrombin is similar in size and conformation to $\alpha$-thrombin except that it does not bind to the thrombin aptamers.[44,45] As shown in Figure 4C, the standardized $T_p$ values obtained for $\gamma$-thrombin (at a concentration of 100 nM) were comparable to those of the negative control sample and clearly at higher values than for $\alpha$-thrombin at 100 pM. Thus, we confirmed (i) the limit-of-detection of 100 pM for $\alpha$-thrombin and (ii) that $\gamma$-thrombin was not forming a stable sandwich by binding to the aptamers. The main source of variability of this quantification assay was the dumbbell quantity forming a sandwich with the target and its amplification. Therefore, the positive control composed of the dumbbell calibration range is necessary for each assay.

Our aptamero-LAMP assay performances were the following for the detection of thrombin: a limit of detection of 100 pM, a limit of quantification of 1 nM, and a dynamic range of 3 orders of magnitude matching the physiological conditions.[42,43] The main limitation of this aptamero-LAMP assay consists of the nonspecific signal. This may be due to unspecific absorption of dumbbells on the magnetic beads' surface. In further developments, it would be interesting to analyze other coatings than the BSA blocking agent on magnetic beads. In this respect, the use of PEG molecules has been shown to improve the aptamer−protein interactions with the advantage to limit the nonspecific interactions.[33,40]

Aptamero-LAMP is not limited to thrombin detection. Many aptamers have been selected toward proteins or other biomarkers.[46,47] Generally, aptamers present similar affinities to antibodies and thus could be an interesting alternative to replace antibodies. In particular, aptamers may be produced easily by chemical synthesis at lengths compatible with the dumbbells (around 150 bases), while antibodies are only biologically produced leading to batch-to-batch variability.[17,48,49] Furthermore, aptamers present several other advantages such as being more robust and less expensive. They are also more suitable for use in point-of-care (POC) devices. On the integration side, the isothermal amplification also presents interesting performances in microfluidic or POC devices.[25,50]

The aptamero-LAMP presented in this study relied on DNA probes. For the detection of oligonucleotide targets, those probes are straightforward to select by simple sequence complementarity. For protein biomarkers, the probes are relying on the selection of a couple of aptamers forming a sandwich with the target. Fortunately, aptamer selection for lots of the biomarkers have been performed. However, only for some cases like for thrombin, two aptamers exist and bind to different epitopes of the target. In the other cases, the selected aptamer should be included in the dumbbell while another kind of probe like antibodies or molecular imprinted polymers should be coated onto the magnetic beads.

## CONCLUSION

We have demonstrated the proof of concept of the aptamero-LAMP assay for both target DNA and thrombin detection. On the basis of an aptamer sandwich binding of the target, we have developed an original LAMP amplification of an oligonucleotide dumbbell structure incorporating an aptamer: the isothermal dumbbell exponential amplification with only two primers. Various analytes can be detected using this method. As a proof of concept, we demonstrated that quantification of a model single strand oligonucleotide can be successfully performed. The dynamic range spreads over 3 orders of magnitude, with a limit of detection at 1 pM. For the detection of thrombin, a limit of detection of 100 pM was observed with a dynamic range of 3 orders of magnitude matching the physiological conditions. Finally, our method appears to be a promising method to perform various biomarkers' quantification with high specificity and sensitivity, using clinical samples in POC situations.

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https://doi.org/10.1021/acs.analchem.1c05532
*Anal. Chem.* 2022, 94, 3376−3385

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

The authors benefited from the financial support of LABEX ARCANE through the EUR CBH-EUR-GS (ANR-17-EURE-0003) and of the Auvergne Rhône Alpes region through the project DEDICATE. We also would like to thank Mélissa Baqué and Anne-Gaëlle Bourdat for fruitful discussions and for the help on the LAMP mix solution optimization.

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https://doi.org/10.1021/acs.analchem.1c05532
*Anal. Chem.* 2022, 94, 3376−3385