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Short communication

# Rapid and specific DNA detection by magnetic field-enhanced agglutination assay

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ABSTRACT

The detection of DNA molecules by agglutination assays has suffered from a lack of specificity. The specificity can be improved by introducing a hybridization step with a specific probe. We developed a setting that captured biotinylated DNA targets between magnetic nanoparticles (MNPs) grafted with tetrathiolated probes and anti-biotin antibodies. The agglutination assay was enhanced using a series of magnetization cycles. This setting allowed to successfully detect a synthetic single stranded DNA with a sensitivity as low as 9 pM. We next adapted this setting to the detection of PCR products. We first developed an asymmetric pan-flavivirus amplification. Then, we demonstrated its ability to detect dengue virus with a limit of detection of 100 $TCID_{50}$/mL. This magnetic field-enhanced agglutination assay is an endpoint readout, which benefits from the advantages of using nanoparticles that result in particular from a very reduced duration of the test; in our case it lasts less than 5 min. This approach provides a solution to develop new generation platforms for molecular diagnostics.

## 1. Introduction

Nucleic Acid Testing (NAT) is commonly used for many diagnostic assays in various fields including genetic diseases, cancer or infectiology [1,2]. This approach requires several sequential steps: nucleic acid extraction, amplification and detection of molecular targets. The last two steps are usually performed with sophisticated thermal cyclers with fluorescence detection by skilled personnel and in a dedicated environment for molecular biology, which is not compatible with point-of-care testing [2–4]. However, various approaches are currently being tested to simplify the amplification step including isothermal molecular amplification techniques such as rolling circle amplification (RCA), loop-mediated amplification (LAMP) or recombinase polymerase amplification (RPA) [2,5–8]. Current detection of molecular targets uses fluorescence detection, but simple optical or electrochemical techniques with a rapid response are under development for biosensor applications [9–13]. Moreover, the detection step could also benefit from the use of magnetic nanoparticles (MNPs) which are one of the most effective strategies for lowering detection limits and nonspecific effects [14,15].

In order to develop a fast and easy-to-use DNA detection step after amplification, we have tested if a magnetic field-enhanced agglutination assay (MFEA assay) could be applied [16,17]. Briefly, this technique consists in applying a magnetic field generated by an electromagnet to the reaction medium to accelerate the capture of the target between MNPs by a fast chaining process (Fig. 1a). The result of this agglutination performed in a homogeneous phase can then be assayed by a simple

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turbidimetry readout in less than 5 min. The design of the DNA agglutination assay can be as simple as biotinylated double stranded DNA targets incubated with streptavidin-linked MNPs (Fig. 1b). However, many situations require DNA sequences to be discriminated from non specific amplification or DNA sequences of high homology, such as identifying a viral strain among a family of viruses. To gain in specificity, a probe can be added to the assay, modifying the design of the chaining process with a third component, the probe-linked MNPs (Fig. 1c). To gain in signal intensity, probes were grafted onto MNPs through a tetrathiolated link. These tetrathiolated probes have been previously evaluated in a microplate format and performed better in detecting viral genomes than ester link probes [19,20]. The biotinylated single stranded DNA is detected using a couple of MNPs on which probes and anti-biotin antibodies have been grafted (Fig. 1a, c).

We report here the development of a simple and rapid magnetic field-enhanced agglutination assay that detects PCR amplified products. The assay, designed to detect dengue virus, has been evaluated to determine its sensitivity and its specificity.

## 2. Material and methods

### 2.1. Design of tetrathiolated DENV probe and grafting magnetic nanoparticles

A generic 15-mer tetrathiolated DENV probe aimed at detecting dengue viral genomes was designed after aligning the nucleotide sequences of the *NS5* gene from 53 strains of DENV, as previously described [18]. The 5′-tetrathiolated DENV probe 5′TCC TTC YAC TCC RCT3′ was synthesized on a 1 μmol-scale using a DNA synthesizer, and lyophilized before use [18,19]. This DENV probe was covalently grafted on 200 nm diameter MNPs (200 nm carboxyl-adembeads, Ademtech, Pessac France). Ademtech manufactures calibrated particles (CV<20%), with high magnetic content (70% of iron oxide) and controlled surface bearing various functionalities. The 200 nm diameter nm carboxyl-adembeads, have been selected in the MFEA assay. These MNPs are monodispersed and super-paramagnetic beads composed of magnetic core encapsulated by a highly crosslinked hydrophilic polymer shell. Briefly, after washing and resuspension in Activating Buffer (AB) 1X (Ademtech, Pessac, France), 11.5 mg of MNPs were incubated for 30 min at 37 °C under agitation at 1000 rpm (ThermoMixer comfort, Eppendorf, Hamburg, Germany) with 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride (6 mg/mL) to form an ester active intermediate. Then, the activated MNPs were incubated with amino-PolyEthyleneGlycol (PEG)-maleimide (8 mg/mL) in AB 1X for 2 h at 37 °C under agitation at 1000 rpm (ThemoMixer comfort). In parallel, 200 nmol of lyophilized polythiolated DENV probe were incubated for 10 min at 20 °C with 100 μL of tris(2-carboxyethyl)phosphine hydrochloride (20 mM) to reduce the disulfide bonds, and 900 μL of Binding Buffer (0.1 M $Na_2HPO_4$, 0.15 M NaCl, 10 mM EDTA, pH7.2) was added. After washing with Storage Buffer (SB) 1X (Ademtech, Pessac, France), the PEG-maleimide MNPs were incubated for 3 h at 20 °C with the reduced polythiolated DENV probe (200 nmol/mL). The beads were placed on a magnet (Ademtech, Pessac, France), to remove the supernatant and were passivated by sequential incubations with 1 mL of tris HCl 1.5 M pH 8.8 for 20 min and 250 μL of a cysteine solution (80 mg/mL) for 10 min. After this blocking step, the MNPs covalently grafted with the DENV probe (MNPs-Probe) were washed twice in 1 mL of SB and stored at 1% w/v in a dedicated buffer (10 mM Glycine 0.02% $NaN_3$, 0.1% F108, pH 9) for up to 6 months at 4 °C.

### 2.2. Magnetic field-enhanced agglutination assay

The prototype included a disposable spectrophotometric cuvette surrounded by an electromagnet that provided a 15 mT (mT) field, a LED source emitting at 650 nm and a photodiode [17]. MNPs grafted with anti-biotin antibodies (MNPs-Ab) were prepared using a carbodiimide coupling chemistry by adding 10 μg of anti-biotin antibody (Jackson ImmunoResearch Europe LTD, Cambridge, UK) to 1 mg of MNPs. Increasing the antibody/MNPs ratio had no impact on the signal. Three cycles of magnetization (60 s) and relaxation (30 s) led to the progressive formation of aggregates, a program previously optimized for protein detection. The turbidity signal was expressed as the total variation of optical density at 650 nm ($\Delta\ OD_{650nm}$) measured before and after the three magnetization cycles.

### 2.3. Detection of synthetic DENV DNA sequences

A synthetic 15-mer DENV DNA oligonucleotide (AGY GGA GTR GAA GGA) biotinylated at its 5′-end (Eurogentec, Angers, France) which was complementary to the DENV tetrathiolated probe, was diluted from 5000 to 0.1 pM in Hybridization Buffer (HB) (6X SSPE, 5X Denhardt solution). A non-complementary 15-mer Zika virus (ZIKV) DNA oligonucleotide (AGC AAG GGG AAT TTG) biotinylated at its 5′-end (Eurogentec, Angers, France) was used to control the non-specific events. The

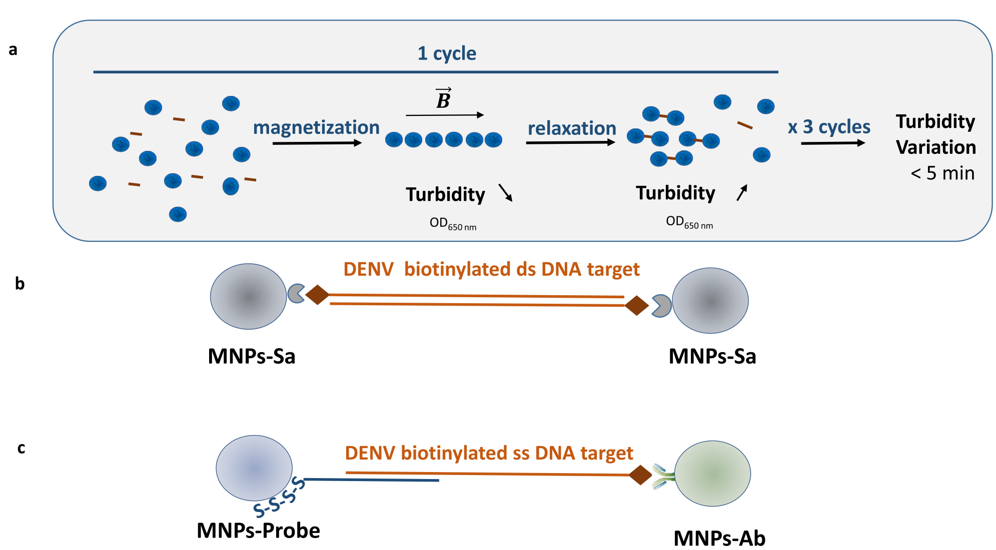



**Fig. 1.** (a) Magnetic field-enhanced agglutination assay. (b) Schematic representation of capture of DENV biotinylated double stranded DNA (ds DNA) target between a pair of magnetic nanoparticles grafted with streptavidin (MNPs-Sa). (c) Schematic representation of capture of DENV biotinylated single stranded DNA (ss DNA) target between MNPs grafted with the specific tetrathiolated DENV probe (MNPs-Probe) and MNPs anti-biotin antibodies (MNPs-Ab).

synthetic DNA sequences were first captured on MNPs-Probe and two readout methods were performed.

Using a time-resolved fluorescence method, MNPs-Probe were incubated with synthetic 15-mer DNA oligonucleotides diluted in (HB) for 5 min at 37 °C in a microtube. The microtube was placed on a magnet to remove the supernatant and was washed three times in SSPE 5X, SDS 0.1% and once in PBS 1X Tween 0.01%. The beads were then incubated for 30 min at room temperature with europium-labeled streptavidin and washed three times in a commercial washing buffer (DELFIA, PerkinElmer, Boston, USA). After the addition of the enhancement solution (DELFIA, PerkinElmer, Boston, USA) and transfer to a microwell, the molecular hybridization events were detected by time-resolved fluorescence using a microplate reader (Victor Instrument, PerkinElmer, Boston, USA). The limit of detection was established by determining the mean value of blank samples plus three times the standard deviation to provide a more conservative detection [20].

Using the MFEA assay, 160 μL of oligonucleotides were incubated for 5 min at 37 °C under agitation with 3 μL of MNPs-Probe (1% w/v). The mix was then transferred into two disposable cuvettes, each containing 72.5 μL of mix and 1.5 μL of MNPs-Ab (1% w/v), to perform the measurement in duplicate. Three pulse cycles of 60 s of magnetization and 30 s of relaxation were applied and the aggregation of MNPs was monitored in real time at 650 nm for 5 min.

The limit of detection was established by determining the mean value of blank samples plus three times the standard deviation [20].

### 2.4. Detection of DENV amplified genomes

Whole flaviviruses (DENV and ZIKV) were provided as reference material by the National Surveillance Center of Arboviruses in Marseille, France. They were supplied as frozen vials consisting of ten-fold serial dilutions of supernatants from infected cell cultures. Ten replicates of each dilution of DENV serotype 1 from 1000 to 10 $TCID_{50}$/mL were used as a DENV model to determine the analytical performance of the MFEA readout. Human plasma samples from blood donors collected by the Etablissement Français du Sang (EFS) in Montpellier (France) with no history of viral infections were used as negative plasma samples.

Viral nucleic acid extraction was performed using the MagNA Pure Compact automated system with the MagNA Pure Compact Nucleic Acid Isolation Kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Entire process of extraction and purification took 30 min. The purified viral nucleic acids were aliquoted and stored at −80 °C until their use.

The MAMD/cFD2 primer pair previously described [21] was used for pan-flavivirus one-step RT-PCR amplification (Qiagen, Valencia, CA, USA) targeting the flavivirus *NS5* gene. The forward primer MAMD (5′AAC ATG GGR AAR AGR GAR AA3′) was 5′-tagged with biotin to generate, after PCR amplification, biotinylated viral genomes. The sequence of the reverse primer cFD2 was 5′GTG TCC CAG CCG GCG GTG TCA GC3'. In order to generate single-stranded biotinylated DNA, an asymmetric PCR amplification was carried out using 5 μL of extracted viral RNAs mixed with 3 μL of biotinylated forward primer (10 μM) and 0.3 μL of reverse primer (10 μM) in a final volume of 50 μL. The RT-PCR conditions consisted of a 30 min reverse transcription step at 50 °C and a 15 min Taq polymerase activation step at 95 °C, followed by an initial denaturation at 95 °C for 5 min, then 40 cycles of 95 °C for 40 s (denaturation), 56 °C for 40 s (annealing) and 72 °C for 1 min (extension), followed by a final extension step of 72 °C for 10 min. The total amplification time lasted 2.5 h. The PCR procedures were performed using a T Advanced Biometra thermal cycler (Analytik Jena AG, Germany) and amplified products were tested immediately or stored at −20 °C until their use. Three readout methods were compared. Amplicons were analysed directly using electrophoresis on a 2% agarose gel, or 1:10 diluted, captured on the MNPs-Probe and tested using the MFEA assay in 2.2 or the fluorescence plate method described in 2.3.

In order to test the performance of the MFEA assay, PCR products were diluted 1:10 in HB, were denatured for 10 min at 95 °C and then placed on ice 5 min before incubation with 3,3 μL of MNPs-Probe for 5 min at 37 °C under agitation. This mix (72.5 μL) was then transferred into two disposable cuvettes containing 1.5 μL of MNPs-Ab (1% w/v) to perform the agglutination assay as previously described. All measurements were performed in duplicate. Synthetic 15-mer DENV DNA oligonucleotides biotinylated at their 5′-end were used at 1000 pM as positive controls in each assay. The limit of detection was established by determining the mean value of blank samples plus three times the standard deviation for a more conservative detection.

## 3. Results

### 3.1. Detection of synthetic DENV DNA sequences

Synthetic single-stranded DNA sequences were captured on the MNPs-Probe and analysed using two different readouts, a fluorescence method (Fig. 2a) or a turbidimetric assay after magnetic field-enhanced agglutination (Fig. 2b). The fluorescent signal increased with the concentration of synthetic DENV DNA sequences up to 1250 pM and showed a low limit of detection of 0.1 pM (Fig. 2a). The turbidity variation ($\Delta$ $OD_{650nm}$) increased with the concentration of synthetic DNA sequences up to 1250 pM and then plateaued. A limit of detection of 9 pM of synthetic DNA was observed (Fig. 2b). The coefficient of variability (CV) of the MFEA test studied on ten assays using synthetic DENV DNA at 1000 pM was 12.35% with a mean $\Delta$ $OD_{650\ nm}$ of 50.10 mOD. The CV observed on ten blank samples was 12.07% with a mean $\Delta$ $OD_{650\ nm}$ of 12.81 mOD. The signal with the synthetic non-specific DNA is close to that obtained with the HB alone in both readouts.

### 3.2. Detection of DENV amplified genomes

In order to apply this readout to the detection of PCR products and to get closer to the above conditions, we have implemented an asymmetric PCR. The turbidity variation is detectable and is proportional to the concentration of virus (Fig. 3a). The limit of detection [20], defined as the concentration giving a signal distinguishable from the analytical noise in the absence of analyte, was 100 $TCID_{50}$/mL for the MFEA assay (Figs. 3a), 10 $TCID_{50}$/mL for the fluorescence method (Figs. 3b) and 100 $TCID_{50}$/mL for the gel electrophoresis method (Fig. 3c). The total assay time including extraction, amplification and detection of viral genomes was 3 h, 6 h or 4 h using MFEA, fluorescence or electrophoresis techniques respectively. No signal was observed with either the negative plasma samples or the blanks by the three readouts (Fig. 3a, b, 3c). At a concentration of 100 $TCID_{50}$/mL, the agglutination method showed a CV of 12.01%. In this system, ZIKV genomes amplified with the pan-flavivirus RT-PCR are not detected using MNPs grafted with DENV probe in the MFEA assay. The signal of agglutination with a non-specific ZIKV amplicon from supernatant cultures titred at 100 000 $TCID_{50}$/mL was close to the signal of the blank samples (Fig. 3d).

## 4. Discussion

In this work, we demonstrate that the magnetic field-enhanced agglutination approach based on a fast chaining process of superparamagnetic nanoparticles under a single magnetic field could be adapted to the detection of nucleic targets in a homogenous phase. Here, the assay is based on the specific capture in a sandwich of nucleic targets between pairs of superparamagnetic nanoparticles grafted with nucleic probes and anti-biotin antibodies.

Similar methods have subsequently been developed using the continuous application of a rotating magnetic field that induced modulation of the scattered light intensity when the particle rotated [22,23]. This magnetic readout was then applied to the detection of DNA previously amplified by rolling cycle amplification (RCA) [24–27] or by loop-mediated amplification (LAMP) [28]. The isothermal amplification

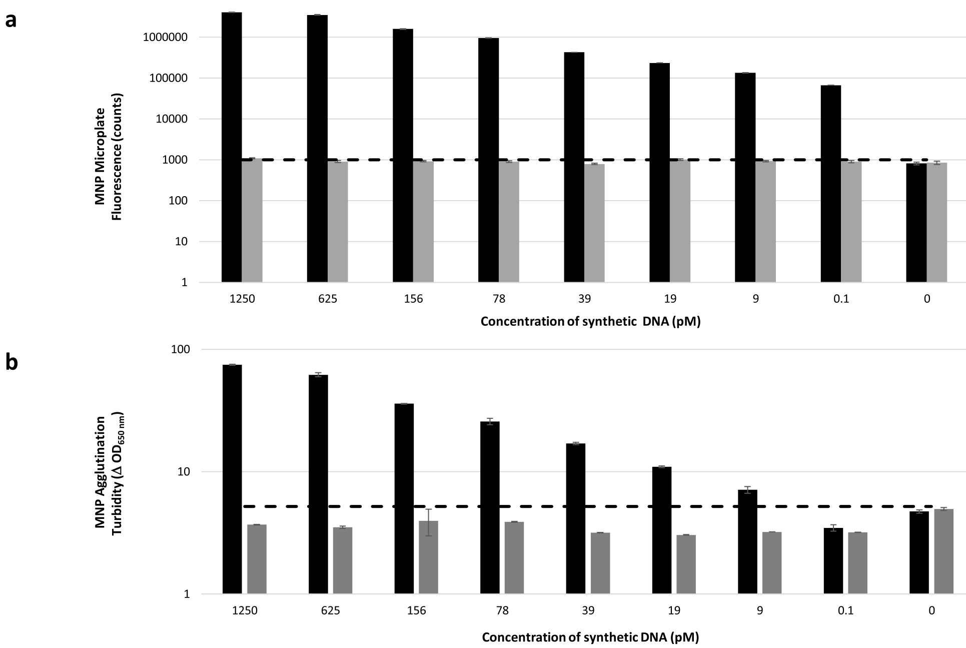
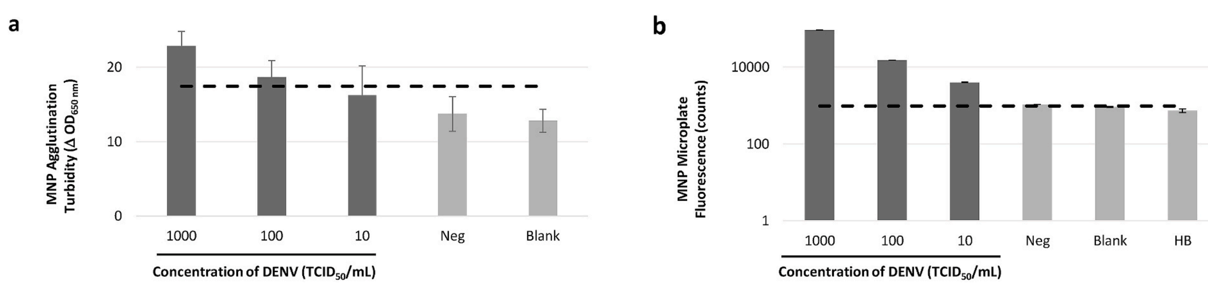
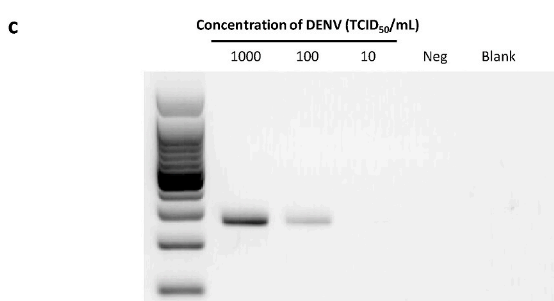



**Fig. 2.** Detection of DENV DNA sequences. Signals are measured for DENV DNA sequences (dark), complementary to the DENV probes grafted on paramagnetic nanoparticles, and for non-specific ZIKV DNA control (grey). Dashed line indicates the limit of detection taken as the mean value of blank samples plus three standard deviations. (a) Fluorescence detection vs synthetic DNA concentration. Data represent two consecutive measurements on the same sample with error bars smaller than the shown data points. (b) Turbidity variation ($\Delta$ $OD_{650\ nm}$) vs synthetic DNA concentration. Each point represents a measurement performed in duplicate. Error bars indicate the standard deviation of two independent measurements for each point.




d

| DENV ($TCID_{50}$/mL) | Mean (n=10) | SD | CV (%) |
|---|---|---|---|
| 1000 | 22.87 | 3.92 | 17.16 |
| 100 | 18.63 | 2.24 | 12.01 |
| 10 | 16.24 | 1.95 | 11.98 |
| Neg | 13.72 | 2.33 | 16.97 |
| blank | 12.81 | 1.55 | 12.07 |
| ZIKV control 100 000 | 13,20 | 1,96 | 14,83 |

**Fig. 3.** Detection of DENV amplified genomes. Signals were analysed for serial dilutions from 1000 to 10 $TCID_{50}$/mL of supernatants from cell cultures infected with DENV. Human plasma samples from blood donors were used as negative plasma samples (Neg). After extraction and amplification using a pan-flavivirus RT-PCR, DENV genomes were analysed using three readout methods. Dashed line indicates the limit of detection taken as the mean value of blank samples plus three standard deviations. (a) MFEA assay. (b) MNPs-Probe based fluorescence method. Hybridization buffer (HB) (c) Electrophoresis on a 2% agarose gel. (d) Coefficients of variation (CV) of the MFEA assay calculated on ten replicates of each sample.

methods present the advantage of avoiding the need for sophisticated thermal cycling platforms. However, their dynamic detection ranges coupled with an optomagnetic readout are limited [28]. In addition, the complexity of primer design particularly in the case of LAMP and of primer ratios for RPA has a significant impact on developing multiplex isothermal amplifications, and optimizations are still needed to better control non-specific molecular events [6,9,28]. The approach we have described herein obviated the discrimination of these non-specific DNA amplifications by introducing a hybridization step to a specific probe. Furthermore, this approach could become very flexible, as specific tetrathiolated probes can be easily substitutable. The limit of detection observed in this first MFEA assay was comparable to gel electrophoresis and one log lower than the microplate time-resolved fluorescent method, but remained compatible with detecting viral loads in clinical samples during the acute phase of many infections.

Detecting molecular targets by the MFEA assay has several advantages: i) the equipment is very simple. It consisted of an optic fiber/ photodiode, an electromagnet and a cuvette holder. The size of the device is as small as a shoe box ii) the handling is minimal and avoids any washing step as classically for techniques with a probe hybridization step, and iii) the “sample in-answer out” detection is very fast with a total time of 5 min compared to 3 h in a microplate format for example [19]. Taken together, these advantages raised the question whether this method could be applied to real time detection, i.e. concomitant with nucleic amplification. Various arguments suggest that it might be possible. First, the detection is carried out in homogeneous phase. The total volume effectively used to detect the turbidimetry is in fact as low as 74 µL. The time of detection could be reduced to 1 cycle lasting less than 10 s, which is compatible with a PCR cycle. The technique also has limitations: as it stands, i) like all readout techniques, it comes after the pre-analytical and amplification steps, which have their incompressible durations; ii) it does not allow multiplexing. Nonetheless, it is noteworthy that these limitations do not hamper the application of this technique to real-time detection.

To date, the detection of DNA molecules by agglutination has suffered from a lack of specificity, which has limited its development. By inserting a probe, which is also responsible for the agglutination process, specificity is now rather well controlled. All the advantages of magnetic nanoparticles use can now be assessed for the detection of nucleic targets, including for the development of easy-to-use molecular systems.

## Credit author statement

Elena Pinchon:Investigation, Methodology, Validation, Writing - original draft; Fanny Leon: Investigation, Methodology, Validation, Writing - original draft; Nevzat Temurok: Investigation, Methodology, Validation; François Morvan: Methodology, Synthesis of tetrathiolated probes, Jean-Jacques Vasseur: Methodology, Synthesis of tetrathiolated probes; Martine Clot: Conceptualization, Funding acquisition; Vincent Foulongne: Conceptualization, Methodology; Jean-François Cantaloube: Methodology, Design of tetrathiolated probes; Philippe Vande Perre: Conceptualization, Funding acquisition; Aurélien Daynès: Conceptualization, Methodology, Supervision, Writing and editing, Funding acquisition; Jean-Pierre Molès: Conceptualization, Methodology, Supervision, Writing and editing, Funding acquisition; Chantal Fournier-Wirth: Conceptualization, Methodology, Supervision, Writing and editing, Funding acquisition.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Nevzat Temurok, Martine Clot and Aurélien Daynes are company employees at HORIBA Medical. François Morvan, Jean Jacques Vasseur, Jean Francois Cantaloube, and Chantal Fournier-Wirth are inventors of patents WO 2013150106 (Thiol compounds and the use thereof for the synthesis of modified oligonucleotides) and WO 2013150122 (Modified oligonucleotides comprising thiol functions and the use of same for the detection of nucleic acids).

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