

Ultrasensitive Real-Time Rolling Circle Amplification Detection Enhanced by Nicking-Induced Tandem-Acting Polymerases

Bo Tian, Jeppe Fock, Gabriel Minero, Francesca Garbarino, and Mikkel Foug Hansen

Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.9b02073 • Publication Date (Web): 27 Jun 2019

Downloaded from <http://pubs.acs.org> on June 28, 2019

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.

Ultrasensitive Real-Time Rolling Circle Amplification Detection Enhanced by Nicking-Induced Tandem-Acting Polymerases

Bo Tian,[†] Jeppe Fock,[‡] Gabriel Antonio S. Minero,[†] Francesca Garbarino,[†] Mikkel Fougth Hansen^{†,*}

[†] Department of Health Technology, Technical University of Denmark, DTU Health Tech, Building 345C, DK-2800 Kongens Lyngby, Denmark

[‡] Blusense Diagnostics ApS, Fruebjergvej 3, DK-2100 Copenhagen, Denmark

* Corresponding author. E-mail address: mfha@dtu.dk

ABSTRACT:

Padlock probe ligation-based rolling circle amplification (RCA) can distinguish single-nucleotide variants, which is promising for the detection of drug-resistance mutations in, *e.g.*, *Mycobacterium tuberculosis* (*Mtb*). However, the clinical application of conventional linear RCA is restricted by its unsatisfactory picomolar-level limit of detection (LOD). Herein, we demonstrate the mechanism of a nicking-enhanced RCA (NickRCA) strategy that allows several polymerases to act simultaneously on the same looped template, generating single-stranded amplicon monomers. Limiting factors of NickRCA are investigated and controlled for higher amplification efficiency. Thereafter, we describe a NickRCA-based magnetic nanoparticle (MNP) dimer formation strategy combined with a real-time optomagnetic sensor monitoring MNP dimers. The proposed methodology is applied for the detection of a common *Mtb* rifampicin-resistance mutation, *rpoB* 531 (TCG/TTG). Without additional operation steps, an LOD of 15 fM target DNA is achieved with a total assay time of *ca.* 100 min. Moreover, the proposed biosensor holds the advantages of single-nucleotide mutation discrimination and the robustness to quantify targets in 10% serum samples. NickRCA produces short single-stranded monomers instead of the DNA coils produced in conventional RCA, which makes it more convenient for downstream operation, immobilization or detection, thus being applicable with different molecular tools and biosensors.

KEYWORDS: tandem-acting polymerases; rolling circle amplifications; *Mycobacterium tuberculosis* detection; magnetic nanoparticles; optomagnetic sensing.

Nucleic acid amplification has played a vital role in molecular biology and clinical diagnostics. The most successful methodology among them, the polymerase chain reaction (PCR), has long been the gold standard in, *e.g.*, epidemiology, forensic science and paleontology. However, limitations of PCR, including the high risk of carry-over contamination and the requirement of a precise thermal cycler, have hampered its applications in today's fast growing field of point-of-care diagnostics. To overcome the practical limitation of PCR, linear and isothermal amplification strategies have been developed and applied in combination with a variety of readout systems.¹⁻³ Among different isothermal amplification techniques, rolling circle amplification (RCA) has attracted significant attention due to its simplicity, versatility and robustness.^{4,5} RCA utilizes circular templates to produce long single-stranded DNA amplicons on solid surfaces, in solution, or even inside cells. RCA products contain programmable repeating units that can be customized for subsequent combination with DNA nanotechnologies and biosensors. In addition, combined with a padlock probe (PLP) ligation process to generate looped templates, RCA-based assays are highly specific and can easily distinguish single-nucleotide variants.⁶⁻⁸ They are thus promising for the detection of, *e.g.*, microRNAs and drug-resistance mutations.

Conventional RCA-based detection methods have picomolar-level limits of detection (LOD), which is unsatisfactory for further clinical applications. Several RCA-based approaches have been developed to improve the sensitivity, either by changing the amplification format from linear to exponential (*e.g.*, hyper-branched RCA⁹ and DNAzyme feedback amplification),¹⁰ or

1
2
3 by performing cascade amplification (*e.g.*, circle-to-circle amplification).¹¹ Exponential
4
5 amplification easily suffers from false-positive results caused by carry-over contamination or
6
7 background products and usually produces double-stranded amplicons, which are less
8
9 convenient for downstream analysis. Cascade amplification always requires additional
10
11 operation steps and sometimes requires different reaction temperatures, which complicate and
12
13 prolong the assay. Therefore, an ultrasensitive and highly specific RCA assay with no additional
14
15 operation processes is highly attractive for point-of-care orientated nucleic acid analysis.
16
17
18
19
20

21
22 Several studies modified RCA by involving DNA nicking reactions (NickRCA) to chip off
23
24 single-stranded amplicons from the looped template for subsequent biosensing or secondary
25
26 amplification.^{12–18} Although not demonstrated, the possibility of nicking-induced tandem
27
28 polymerases acting on a single looped template has been suggested in previous studies.^{15,16}
29
30 However, the designs of these previous NickRCA strategies had three main restrictions that
31
32 limited the NickRCA performance: (a) The looped template employed in conventional RCA
33
34 was *ca.* 70-nt-long (loop diameters of *ca.* 8 nm), which provided only limited space for tandem-
35
36 acting polymerases. (b) Amplicons generated in these designs could be degraded due to the 3'-
37
38 5' exonuclease activity of DNA polymerase,¹⁹ which has a strong influence for low target
39
40 concentrations where most polymerases are not acting on the looped template. (c) Due to the
41
42 high acting speed of polymerases as well as the small size of the loop, many amplicon dimers
43
44 and polymers are generated, which can influence the downstream signal transduction. Herein,
45
46 for the first time we demonstrate the existence of nicking-induced tandem-acting polymerases
47
48 on looped templates and evaluate effects that limit the sensitivity of NickRCA. Moreover, by
49
50 employing a restriction endonuclease (instead of nickases) as well as a blocked amplicon
51
52 protector, our method can generate homogeneous RCA monomers with protected 3'-ends. The
53
54 NickRCA-generated amplicons simultaneously hybridize with detection probes modified
55
56
57
58
59
60

magnetic nanoparticles (MNPs), resulting in the formation of MNP dimers and/or clusters that can be monitored in real-time using an optomagnetic setup.

Tuberculosis, one of the most catastrophic diseases in human history, is a contagious airborne disease caused by the infection of *Mycobacterium tuberculosis* (*Mtb*). World Health Organization estimated in the 2018 global tuberculosis report that about 10 million people had developed tuberculosis and that the disease caused about 1.6 million deaths in 2017.²⁰ In addition, drug-resistant tuberculosis continued to be a public health crisis in 2017, with an estimate of 0.48-0.64 million cases that were resistant to rifampicin, the most effective first-line drug.²⁰ Due to the limitations of current diagnostic techniques (*i.e.*, culture-based, PCR-based, and antigen-antibody reaction-based methods) and the requirements for rapid analysis of *Mtb* drug susceptibility, many efforts have been made during the past few decades towards affordable, rapid and user-friendly point-of-care diagnostics of tuberculosis.^{21,22} In this study, we applied the NickRCA-based optomagnetic DNA biosensor for the detection a common *Mtb* rifampicin-resistance mutation, *rpoB* 531 TCG/TTG genotyped by Engström *et al.*²³ An ultrasensitive limit of detection (LOD) of 15 fM was achieved with a total assay time of approximately 100 min, which is *ca.* two orders of magnitude more sensitive than conventional RCA-based strategies. Additionally, the proposed method can distinguish the single-nucleotide variation between mutated and wild type *Mtb*, which is important for the detection and control of tuberculosis.

METHODS

Chemicals and DNA Sequences. Phi29 polymerase, phi29 buffer, dNTP mix, bovine serum albumin (BSA), GeneRuler low range DNA ladder, and Tris-HCl buffer (1 M, pH 8.0) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ampligase and AmpL buffer

were purchased from Nordic Biolabs (Täby, Sweden). AluI restriction endonuclease was purchased from New England BioLabs (Ipswich, MA, USA). Fetal bovine serum (FBS), Tris-borate-EDTA (TBE, 10×) buffer, and agarose were purchased from Sigma-Aldrich (St. Louis, MO, USA). EZ-Vision dye-as-loading buffer was purchased from VWR (Radnor, PA, USA). Streptavidin-coated cross-linked starch iron oxide composite particles (100 nm size MNP, 10 mg/mL, product code 10-19-102) were purchased from Micromod Partikeltechnologie GmbH (Rostock, Germany). DNA sequences were synthesized by Integrated DNA Technologies (Coralville, IA, USA) and diluted in 50 mM Tris-HCl (pH 8.0). Sequences of *Mtb rpoB* with rifampicin-resistance mutation (target DNA of the RCA reaction), wild type *Mtb rpoB* (single-nucleotide mismatch DNA used in the specificity test), padlock probes (PLPa, PLPb and PLPc), detection probes (DP-1 and DP-2), and the amplicon protector (also served as the AluI restriction oligo) are listed in **Table S1**. Synthetic non-target DNA sequences used for the specificity test are listed in **Table S2**.

Functionalization of Magnetic Nanoparticles. Tris-HCl (50 mM, pH 8.0) was used for MNP washing (using a magnetic separation stand) and resuspension. Streptavidin-coated 100 nm MNPs were washed twice and resuspended to 1 mg/mL before conjugation. Biotinylated detection probes were added into the MNP suspension to a concentration of 0.25 μ M. The suspension was incubated at 37°C for 30 min, washed 3 times to remove unbound detection probes, and resuspended to a nanoparticle concentration of 1 mg/mL. DP-1 modified MNPs and DP-2 modified MNPs were prepared separately, mixed in a volumetric ratio of 1:1, and stored at 4°C prior to use.

Padlock Probe Ligation and Rolling Circle Amplification. The ligation mixture (20 nM) consisted of AmpL buffer (1×), BSA (0.2 μ g/ μ L), PLP (60 nM), target DNA (20 nM), and

Ampligase (0.25 U/ μ L). The ligation mixture was incubated at 55°C for 10 min to prepare looped templates (20 nM, with on-loop target DNAs). Thereafter, the ligation mixture was diluted with 50 mM Tris-HCl (pH 8.0) to obtain different concentrations of looped templates (with on-loop target DNAs). The RCA reaction mixture for conventional RCA (*i.e.*, no nicking) consisted of phi29 buffer (2 $\times$), BSA (0.4 μ g/ μ L), dNTPs (0.45 mM), phi29 polymerase (0.67 U/ μ L), and detection probe-modified MNPs (0.1 mg/mL). For NickRCA, amplicon protector and AluI were added to the RCA reaction mixture to concentrations of 2 μ M and 0.25 U/ μ L, respectively. The RCA reaction mixture was mixed with (diluted) ligation mixture in a volumetric ratio of 1:1, followed by isothermal incubation at 38°C on chips (for real-time optomagnetic measurement) or on a ThermoShaker incubator (Grant-bio, Cambridge, UK).

Post-Monomerized Rolling Circle Amplification. For post-monomerized RCA, a polymerase inactivation step (65°C for 10 min) was performed after conventional RCA (without MNPs). After cooling down, amplicon protector and AluI were added to the inactivated RCA suspension for a monomerization process (38°C for 30 min). The same volume of water was added to the NickRCA suspension to adjust the amplicon concentration before comparing post-monomerized RCA and NickRCA in gel electrophoresis.

Real-Time Optomagnetic Measurement with Magnetic Incubation. A detailed description of the optomagnetic sensor is provided in **Supplementary Section S1**. Optomagnetic detection chips (a minimum detection volume of 90 μ L) containing RCA suspensions were sealed and thereafter mounted in the optomagnetic setup. Ten magnetic incubation cycles were automatically performed between two optomagnetic measurements, with each cycle consisting of 2 s of 2.6 mT field followed by 2 s of 0 mT. For optimization of magnetic incubation periods, 40 optomagnetic measurements were performed during *ca.* 60 min of real-time detection. For

dose-response detections and specificity tests, 60 optomagnetic measurements were performed during *ca.* 90 min of real-time detection.

Agarose Gel Electrophoresis. To verify the products of NickRCA, demonstrate the influence of PLP lengths and investigate limiting effects of amplification efficiency, products of NickRCA and post-monomerized RCA were mixed with dye-as-loading buffer and analyzed by 1% (w/v) agarose gel electrophoresis in 1× TBE buffer at a constant voltage of 50 V for 20 min at room temperature. Gel images were obtained by using a UVP BioSpectrum Imaging System (Analytik Jena, Jena, Germany).

RESULTS AND DISCUSSION

Molecular Amplification Principle. The nicking-induced tandem-acting polymerase RCA (illustrated in **Figure 1**) combines a conventional RCA reaction with a restriction endonuclease-based nicking reaction. The PLPs, *i.e.*, the looped templates, are designed with one or two cutting-sites (nucleotide sequence of AGCT, illustrated as the red sequence in **Figure 1**) that are protected by the 5-methylated deoxycytidine. Due to the methylation, the restriction endonuclease (AluI utilized in this study) can cleave the amplicon strand but not the template strand in the amplicon-template double strands, resulting in a nicking effect. Due to the high polymerizing efficiency as well as the strand displacement ability of phi29 polymerase used in this study, amplicons can release from the template due to the circling of phi29 without being fully nicked, to generate amplicon dimers and polymers. In addition, phi29 has a 3'-5' exonuclease activity that can degrade single-stranded amplicons chipped off from the looped template, which could strongly influence the amplification results especially when the target concentration is much lower than the phi29 concentration. To overcome the abovementioned two issues, a 3'-end blocked 25-nt-long protector was added in the NickRCA reaction. The

protector hybridized with the 3'-end zone of the amplicons to prevent exonucleolytic digestion by phi29. Moreover, the 5'-end of the protector has a 6-nt-long overhang covering an AluI cutting-site, which makes it serve also as a restriction oligo that can hybridize to the intact cutting-sites in the middle of amplicon dimers/polymers and induces the off-loop cleavage to produce amplicon monomers. The amplicon protector functions are illustrated in **Figure 1**.

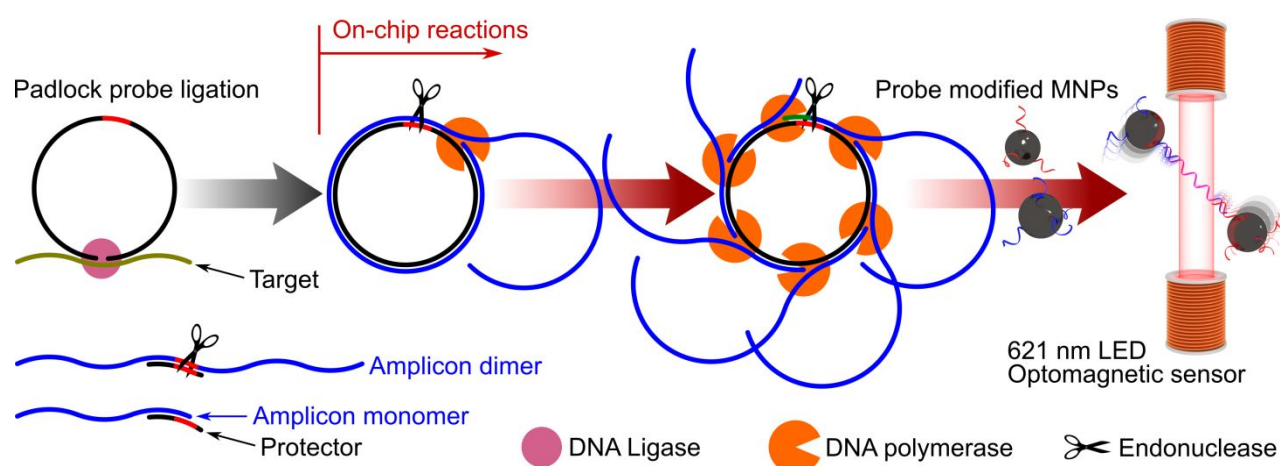


Figure 1. Schematic illustration of PLP ligation, nicking-induced tandem-acting polymerase RCA, and real-time optomagnetic detection of amplicon monomers. NickRCA employs several polymerases to act simultaneously on the same looped padlock probe to generate single-stranded amplicon monomers that lead to the formation of MNP dimers/clusters. Aggregated MNPs are monitored by an optomagnetic setup. NickRCA, probe modified MNP hybridization, and optomagnetic detection take place simultaneously on-chip at 38°C. The amplicon protector functions are illustrated at bottom left.

In practice, an excess amount of PLP will be utilized in the ligation step to hybridize with the target, resulting in the existence of linear PLPs in the RCA suspension that can hybridize with amplicon monomers and trigger a secondary amplification further increasing the amplification efficiency. In this study, the working concentration of protector (1 μM) is more than ten

thousand times higher than the concentration of linear PLPs in the RCA suspension (30 pM at most), hence the influence of secondary amplification is negligible due to competitive binding.

Tandem-Acting Polymerase and Limitations of NickRCA. The on-loop nicking reaction converts on-loop amplicons into new primers, attracting idle polymerases to start new RCA reactions resulting in tandem-acting polymerases on a single loop. The phenomenon of nicking-induced tandem-acting polymerases was suggested in previous studies but not demonstrated.^{15,16} The size of phi29 (67 kDa) can be simply estimated as a sphere with a diameter of *ca.* 4 nm. Previously reported NickRCA employed around 70-nt-long PLPs, *i.e.*, loops with diameters of *ca.* 8 nm. Considering that looped templates are not perfectly circular in solution and phi29 needs additional space to perform polymerization, we hypothesize that the number of tandem-acting polymerases on each looped template is likely to be strongly influenced by the PLP length. Therefore, the existence of tandem-acting polymerases can be evidenced by demonstrating the PLP-size-dependence of NickRCA efficiency.

To demonstrate the influence of PLP size as well as the number of nicking sites, we designed three different PLPs. For PLPa, PLPb and PLPc, the lengths were 100, 200 and 200 nt, and the numbers of nicking sites were 1, 1 and 2, respectively (illustrated in **Figure 2a**). The target DNA was mixed with a 3-fold excess amount of PLPs in the ligation step and diluted after ligation to make sure the concentrations of different looped PLPs were comparable. The working concentrations of looped PLP in the RCA suspension were indicated in **Figure 2**. The NickRCA products generated by different working concentrations (*i.e.*, 0.4, 0.2 and 0.1 nM) of these looped PLPs were studied by gel electrophoresis. As shown in **Figure 2a**, the 200-nt-long PLPb and PLPc generated approximately four times more amplicons than the 100-nt-long PLPa (counted by the total number of incorporated nucleotides but not the copies of amplicons),

1
2
3 which evidenced the existence of tandem-acting polymerases. We have also excluded the
4
5 possibility that the PLP length influenced the phi29 polymerizing rate (**Figure S2**). Although
6
7 Huang *et al.* claimed that multiple nicking sites could lower the detection limit probably by
8
9 increasing the possibility of nicking reactions,¹⁵ we did not observe any significant difference
10
11 in amplification efficiency between NickRCAs using PLPb and PLPc. To demonstrate the
12
13 improvement of NickRCA in amplification efficiency, we monomerized the conventional-
14
15 RCA-produced amplicon coils by post-addition of restriction endonuclease (AluI) as well as
16
17 restriction oligos (*i.e.*, post-monomerized RCA or RCA-Cut for simplicity), and compared the
18
19 results to NickRCA products. Gel electrophoresis results (**Figure 2b**) show that NickRCA was
20
21 approximately four times more effective than RCA-Cut, implying a better sensitivity of PLPc-
22
23 based NickRCA assays. Since nearly no amplicon dimers or polymers were observed in
24
25 NickRCA and RCA-Cut reactions by electrophoresis, we confirmed that RCA products were
26
27 fully monomerized, and therefore the stained components on the top of the electrophoresis lanes
28
29 were not amplicons but dNTPs and BSA (**Figure S3**).
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

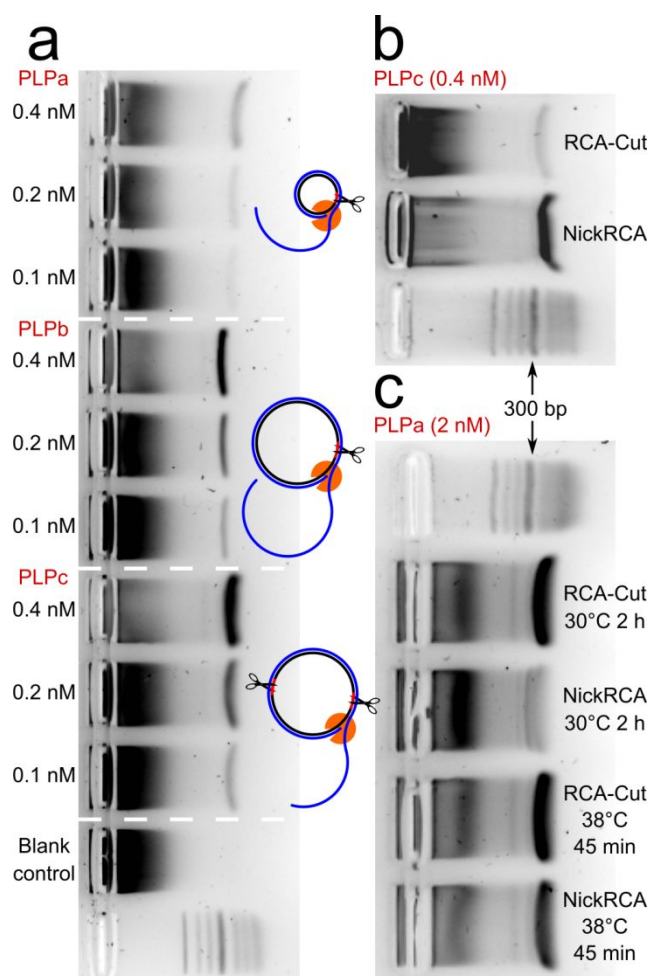


Figure 2. Agarose gel electrophoresis analysis. (a) NickRCA products by using different PLPs. (b) Comparison of PLPc-based NickRCA and post-monomerized RCA. (c) Comparison of PLPa-based NickRCA and post-monomerized RCA at different RCA conditions.

Providing that PLPc-based NickRCA was *ca.* four times more effective than both PLPa-based NickRCA (cf. **Figure 2a**) and RCA-Cut (cf. **Figure 2b**), we have encountered a question: Why does PLPa-based NickRCA not significantly improve the amplification efficiency compared to RCA-Cut? Here we formed two hypotheses: (a) Working endonucleases can stop the movement of on-loop polymerases, making them stuck in a ‘traffic jam’. (b) Nicking reactions produce on-loop amplicons that have a 5'-end at the nicking site and a 3'-end extending by the latest polymerase that passed the nicking site (the green sequence in **Figure 1**), which can be too short to stay hybridized on the loop. Both of these two supposed effects are sensitive to the reaction

time of a single nicking action. On the one hand, a longer reaction time of a single nicking action leads to stronger ‘traffic jam’ effect and that decreases the amplification efficiency. On the other hand, a longer reaction time of a single nicking action means that the polymerase passing the nicking site has more time to synthesize a long enough product to remain stable on the loop. In addition, if a longer reaction time of a single nicking action is achieved by lowering the reaction temperature, the stability of on-loop amplicons will further increase due to the lower thermal energy.

To evaluate the dominating effect in the PLPa-based NickRCA, we performed both NickRCA and RCA-Cut reactions at two different conditions: at 38°C for 45 min or at 30°C for 2 h (see **Figure 2c**). Electrophoresis analysis shows that the RCA-Cut reactions performed in those two conditions produced similar amounts of amplicons, suggesting that polymerases passed the nicking site a similar number of times in the two reactions (polymerization faster for a shorter duration vs slower for a longer duration). Comparing the NickRCA and RCA-Cut results for the two temperatures, it is clear that the efficiency of NickRCA is comparable to that of RCA-Cut at 38°C but significantly reduced at 30°C. These results suggest that longer reaction time of a single nicking action results in lower NickRCA efficiency compared to RCA-Cut, *i.e.*, the ‘traffic jam’ effect dominates the efficiency loss in the PLPa-based NickRCA. This result also explains the low sensitivity of previously reported NickRCA systems.

Optomagnetic Biosensing Principle. Although PLPb- and PLPc-based NickRCA produced similar amounts of incorporated nucleotides, PLPc was chosen for the subsequent biosensing application since it contained two cutting-sites and thus the PLPc-based NickRCA produced twice the amount of amplicon monomers (more but shorter copies) compared to the PLPb-based NickRCA. Except for the components of NickRCA, single-stranded detection probe modified

100 nm sized MNPs were added into the reaction suspension to monitor the NickRCA process. Two different detection probes were used to recognize two different zones of the amplicon monomers, resulting in the formation of amplicon-induced MNP dimers and/or clusters (depending on the concentration of amplicon monomers). The hybridization-induced MNP hydrodynamic size increase was monitored using a previously described 4-chip optomagnetic setup with integrated temperature control.²⁴

A detailed underlying theory of the optomagnetic sensor is provided in **Supplementary Section S1** as well as in our previous work.^{25–28} In the present study, we found that red light emitting diodes ($\lambda = 621$ nm) produced a more sensitive signal than the wavelength of 405 nm used in previous studies.^{29–31} Moreover, we found that the formation of particle dimers and small clusters was best observed as a change of the phase lag φ of the signal, which is insensitive to variations in the MNP concentration.²⁷ Typical time-resolved optomagnetic spectra are shown in **Figure 3**. A blank control sample and a positive control sample were NickRCA amplified as well as measured at 38°C for 60 min. **Figure 3a** and **b** show the typical spectra of the V'_2 and V''_2 optomagnetic signals as well as of the phase lag φ of the magnetic response. The decreasing (and negative) φ -values at frequencies below about 30 Hz (**Figure 3c**) indicate that MNP dimers and/or clusters formed and that these show a signal of opposite sign of the individual MNPs in agreement with previous studies.²⁷ The blank control sample also revealed a slight decrease of φ with time caused by nonspecific MNP aggregation. To focus on the detection of small MNP dimers and clusters that provided the largest φ -decrease at around 10 Hz, we based the analysis on the average φ -value in the interval 3–24 Hz (grey zone in **Figure 3**) denoted as $\overline{\varphi}$.

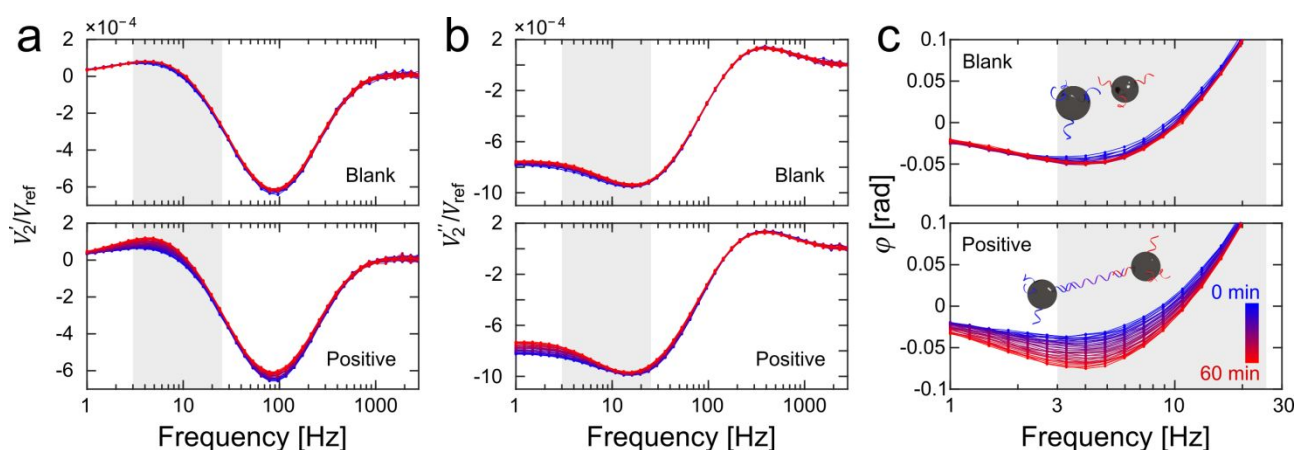


Figure 3. Time-resolved optomagnetic spectra of NickRCA for the detection of a blank control sample and a positive control sample (target concentration of 1 pM) without magnetic incubation. The blue curve indicates the initial spectrum at 0 min while the red curve indicates the final spectrum at 60 min. The grey zone indicates the frequency range used to calculate average measured φ values.

Magnetic Incubation Optimization. Magnetic incubation, *i.e.*, applying magnetic field during the reaction to trigger MNP aggregation, has previously been demonstrated to dramatically accelerate the recognition rate and lower the detection limit.^{32,33} To improve the performance of the proposed NickRCA-based biosensor, magnetic incubation cycles were performed between optomagnetic measurements (see **Supplementary Section S2** and **Figure S1**).

NickRCA-Based DNA Quantification. Time-resolved amplicon traces of NickRCA reactions can provide valuable information that cannot be obtained by simple end-point measurements. Synthetic *Mtb rpoB* (MUT) sequences of different concentrations were quantified in real-time by the optomagnetic sensor using the proposed NickRCA strategy and the conventional RCA strategy, respectively. In the conventional RCA strategy, probe modified MNPs bound to the RCA coils and formed large clusters. Representative φ -spectra of NickRCA-based target detection are shown in **Figure 4**. At the frequency range of 1-30 Hz, aggregation of MNPs

induced a decrease of φ -values in proportion to the target concentration. Furthermore, with the increase of monomer concentration (due to either longer reaction or higher target concentration), MNP clusters were formed following the formation of MNP dimers, which was evidenced by the shift of the φ -valley to lower frequencies.

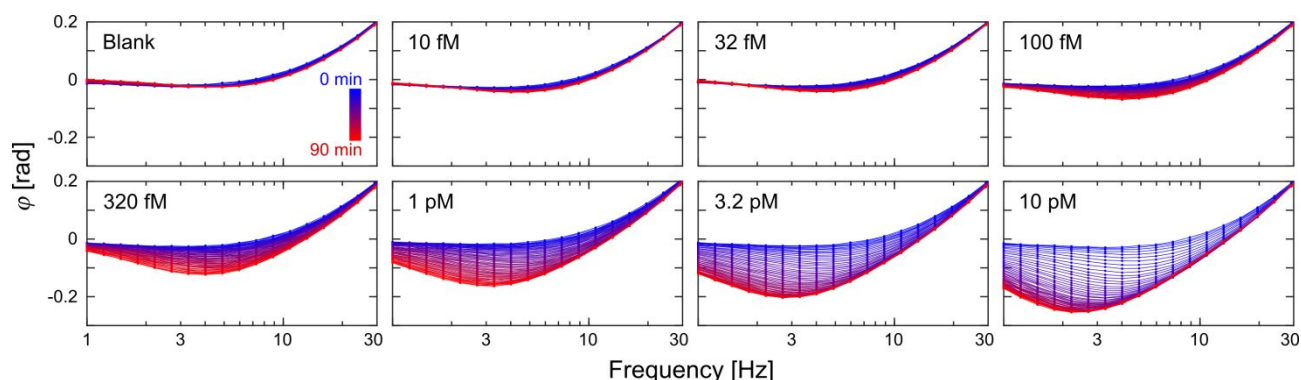


Figure 4. Representative time-resolved φ -spectra of NickRCA-based target detection. The blue curve indicates the initial spectrum at 0 min while the red curve indicates the final spectrum at 90 min. Target concentration in the NickRCA suspension is indicated in the panel.

Figure 5a and b show the real-time signal change, $-\Delta\bar{\varphi}$, from the $\bar{\varphi}$ -value obtained after 8 min of reaction (to exclude the influences of temperature fluctuation and magnetic incubation-induced nonspecific MNP aggregation) during 90 min of NickRCA and conventional RCA reactions, respectively. Amplification reactions with higher target concentrations showed sharper curve slopes and reached the signal plateau earlier. Once the signal plateau was reached, the $-\Delta\bar{\varphi}$ value remained stable, suggesting that the 20-nt-long detection probes lead to MNP aggregates that were robust at 38°C to the competitive binding of redundant amplicon monomers. This confirmed that a monomer-saturation-based hook effect was negligible in this work. However, in the NickRCA-based detection (**Figure 5a**), we observed a negative correlation between the signal plateau values and target concentrations higher than 3 pM, which was ascribed to the fast increasing signals during the first 8 min (used for subtraction) and the

shift of the φ -valley (due to the formation of MNP clusters). In the conventional RCA strategy that produced micron-sized amplicon coils,³⁴ coil-induced MNP clusters could be very large and even larger due to cross-linking between coils (representative φ -spectra are shown in **Figure S4**). As a result, the conventional RCA strategy provided a higher signal plateau than the NickRCA strategy (cf. **Figure 5a** and **b**).

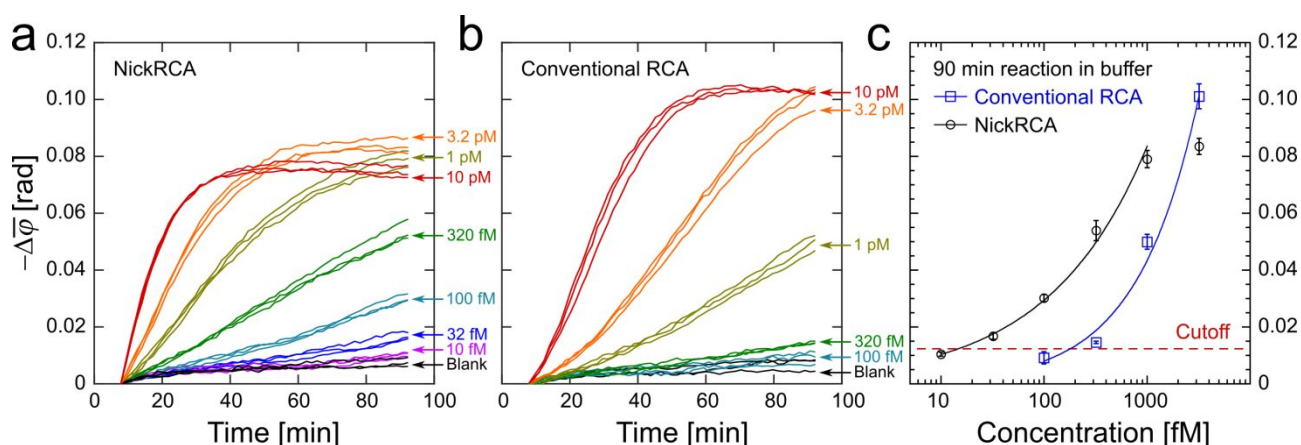


Figure 5. Real-time changes of $\bar{\varphi}$ (3–24 Hz) for different target concentrations amplified by (a) NickRCA and (b) conventional RCA. (c) Dose-response curves of NickRCA-based (black circles) and conventional RCA-based (blue squares) *rpoB* (MUT) detection after 90 min of amplification. The red dashed line indicates the cutoff value calculated based on 3σ criterion. Error bars indicate the standard deviations based on three independent replicates.

The optomagnetic signals obtained after 90 min amplification were selected for the dose-response curves (**Figure 5c**). The cutoff value was calculated as the average signal plus three standard deviations (σ) of the blank controls. For NickRCA-based *rpoB* (MUT) detection, $-\Delta\bar{\varphi}$ had a monotonic positive correlation with the target concentration between 10 fM and 3 pM, with a dynamic detection range of *ca.* 2 orders of magnitude (black circles in **Figure 5c**). An LOD of 15 fM was obtained based on the 3σ criterion. For comparison, the LOD obtained by the conventional RCA reaction (blue squares in **Figure 5c**) was 300 fM. The cutoff value for

the conventional RCA-based strategy (not shown in the plot) was very close to that obtained from the NickRCA-based strategy. Both methodologies exhibited a good repeatability; the average coefficient of variation (CV) for all the measured points in the dynamic detection range was 5.3% for NickRCA and 7.4% for conventional RCA. Comparing to conventional RCA-based detection, the proposed NickRCA-based detection provided a 20 times lower LOD and twice the dynamic detection range, which was ascribed to (a) the increased amplification efficiency due to nicking-induced tandem-acting polymerases and (b) monomer-induced MNP dimer detection strategy (coils produced by conventional RCA are fewer and harder to disperse compared to amplicon monomers). In addition, real-time NickRCA enables tuning of the dynamic detection range and detection limit by simply varying the amplification duration, which also suggests that a lower LOD can be achieved by extending the amplification time.

Conventional RCA was employed for several of end-point magnetic detection systems, typically involving a hybridization step after amplification to form amplicon coil-aggregated MNPs (**Table S3**). For reported end-point magnetic biosensors using RCA coils to aggregate MNPs, LODs of 1, 2, 2, 3 and 4 pM were achieved by a ferromagnetic resonance sensor,³⁵ anisotropic magnetoresistance sensors,³⁶ an optomagnetic sensor,³⁷ a superconducting quantum interference device,³⁸ and an alternating current susceptometer,³⁹ respectively. The similar LODs achieved by these systems indicate that picomolar sensitivity is typical for conventional RCA-based end-point MNP detection strategies. Although NickRCA provided better performance in terms of amplification efficiency and monomer-induced MNP dimer formation, the new strategy could hardly be applied directly in such amplification-hybridization end-point detection due to a strong hook effect (data not shown), since monomer-saturated MNPs prevent aggregation whereas coil-saturated MNPs still provide a strong signal due to the dramatic increase of the hydrodynamic MNP size.

All previously reported NickRCA-based methodologies,^{12–18} except for a methyltransferase detection one, were applied for nucleic acid analysis, thus allowing for direct comparison among them. Sensors, strategies, PLP lengths, amplification durations and LODs of reported NickRCA-based analytical systems are listed in **Table S4**. The most sensitive NickRCA-based methodology, first reported by Taku *et al.*,¹² called primer generation-RCA (PG-RCA) employed prefabricated looped templates to transform NickRCA from linear amplification into exponential amplification. However, due to the exclusion of ligation processes, this attomolar detection limit was achieved at the expense of specificity and in particular the ability to discriminate single-nucleotide mismatches. Compared to other NickRCA-based nucleic acid detection systems, our real-time optomagnetic biosensor provided at least two orders of magnitude improvement in LOD with no additional assay steps. We ascribe this improvement to (a) longer PLPs that allowed several tandem-acting polymerases, (b) utilization of the amplicon protector to prevent the products from the exonucleolytic digestion by phi29, (c) generation of homogeneous amplicon monomers, and (d) the optomagnetic sensing ability to detect MNP dimers.

NickRCA Performance in Serum. It was reported that phi29-based RCA remain functional in 50% human blood,⁴⁰ while Taq DNA polymerase-based PCR was completely inhibited in 0.2% human blood.^{41,42} However, for the proposed strategy, matrix effects influence not only the RCA but also the nicking reaction and MNPs. To evaluate the robustness of the proposed biosensor against matrix effects, target *Mtb rpoB* (MUT) sequences were amplified and detected in samples containing 10% FBS (fetal bovine serum). Real-time optomagnetic spectra showed that FBS influenced neither the efficiency nor the speed of NickRCA (cf. slopes of curves in **Figures 5a** and **6a**) but caused additional nonspecific MNP aggregation, resulting in

a slightly decreased biosensing sensitivity due to the higher cutoff value. According to the dose-response curve shown in **Figure 6b**, a linear detection range from 30 fM to 3 pM was obtained with an LOD of 40 fM. Moreover, matrix effects also decreased the repeatability of NickRCA, reflected as an increased average CV of 8.7%. For the detection of 10% FBS samples, representative φ -spectra are shown in **Figure S5**.

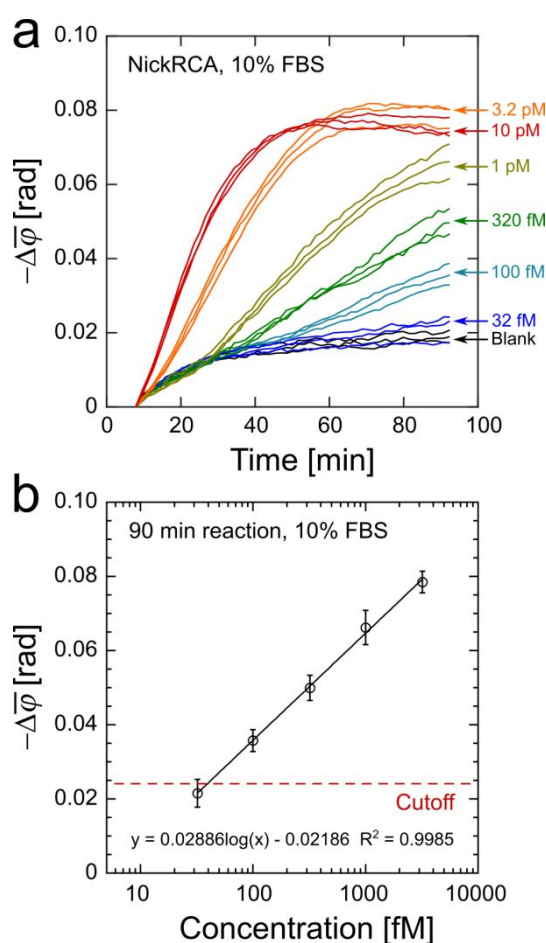


Figure 6. Serum tests. (a) Real-time changes of $\bar{\varphi}$ (3-24 Hz) for different concentrations of target spiked in 10% FBS samples. (b) Dose-response curve for the NickRCA-based optomagnetic quantification of *rpoB* (MUT) in 10% FBS samples. The black solid line and the red dashed line in panel b indicate the linear detection range and the cutoff value, respectively. Error bars indicate the standard deviation based on three independent replicates.

Specificity Test. The primary reason for choosing a PLP ligation-based RCA strategy is the high specificity, which is of high importance in *Mtb* detection for analysis of drug-resistance mutations. The ability of single-nucleotide mismatch discrimination of the proposed biosensor was evaluated. Synthetic non-target DNA sequences (detailed in **Tables S1** and **S2**), including the wild type (WT) *Mtb rpoB* that has only one single-nucleotide mismatch with the PLP target-binding arms (locating at the 3'-end of PLP), were detected. As shown in **Figure S6**, optomagnetic signals of all four non-target DNA sequences are below the cutoff value, indicating a high specificity as well as a capability of discriminating *Mtb rpoB* (MUT) from *Mtb rpoB* (WT).

CONCLUSIONS

In this study, we for the first time demonstrated the existence of nicking-induced tandem-acting polymerases in RCA, investigated the limiting effects of NickRCA, and proposed a more effective NickRCA strategy employing long PLPs and amplicon protectors. Thereafter, we designed a NickRCA-based MNP dimer formation strategy and combined it with a real-time optomagnetic sensor that focused on MNP dimer detection. With no additional operation steps, an LOD of 15 fM was achieved with a total assay time of approximately 100 min for the detection of a rifampicin-resistance mutation in *Mtb rpoB*. Since the proposed NickRCA strategy does not require any additional operation steps compared to conventional RCA, the new findings can be directly utilized to improve the performance of many existing RCA-based methodologies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Optomagnetic sensing principle; magnetic incubation optimization; evaluation of magnetic incubation processes; agarose gel electrophoresis analysis of RCA-Cut products; agarose gel electrophoresis analysis of RCA components; representative time-resolved φ -spectra of conventional RCA-based detection; representative time-resolved φ -spectra of NickRCA-based detection in 10% FBS; specificity test; DNA sequences used in this study; non-relevant DNA sequences used in the specificity test; summary of RCA-based magnetic sensors/biosensors; summary of NickRCA-based methodologies.

AUTHOR INFORMATION

Corresponding Author. *E-mail: mfha@dtu.dk

Notes. The authors declare no competing financial interest.

ACKNOWLEDGEMENT

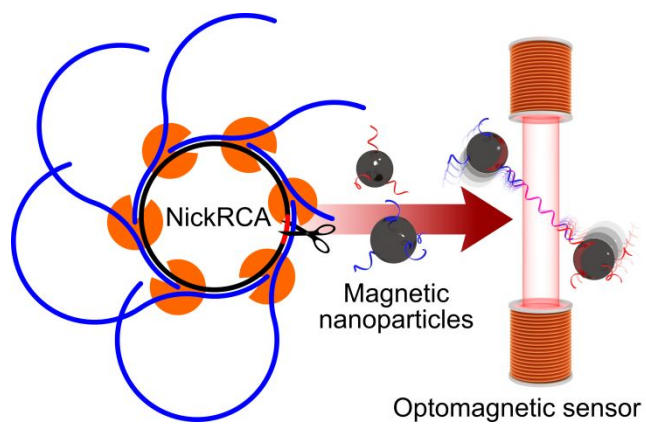
This work was financially supported by H2020 Marie Skłodowska-Curie Actions (Grant No. 713683, COFUNDfellowsDTU), MUDP (Grant No. MST-141-01415) and DFF (Grant No. 4184-00121B). Prof. Mats Nilsson at the Department of Biochemistry and Biophysics, Stockholm University is gratefully acknowledged for discussion on the phenomenon of nicking-induced tandem-acting polymerase.

REFERENCES

- (1) Zhao, Y.; Chen, F.; Li, Q.; Wang, L.; Fan, C. Isothermal Amplification of Nucleic Acids. *Chemical Reviews*. **2015**, *115*, 12491–12545.
- (2) Li, J.; Macdonald, J. Advances in Isothermal Amplification: Novel Strategies Inspired by Biological Processes. *Biosens. Bioelectron.* **2014**, *64*, 196–211.
- (3) Deng, H.; Gao, Z. Bioanalytical Applications of Isothermal Nucleic Acid Amplification Techniques. *Anal. Chim. Acta* **2015**, *853*, 30–45.
- (4) Zhao, W.; Ali, M. M.; Brook, M. A.; Li, Y. Rolling Circle Amplification: Applications in Nanotechnology and Biodetection with Functional Nucleic Acids. *Angew. Chem. Int. Ed.* **2008**, *47*, 6330–6337.
- (5) Ali, M. M.; Li, F.; Zhang, Z.; Zhang, K.; Kang, D. K.; Ankrum, J. A.; Le, X. C.; Zhao, W. Rolling Circle Amplification: A Versatile Tool for Chemical Biology, Materials Science and

- Medicine. *Chem. Soc. Rev.* **2014**, *43*, 3324–3341.
- (6) Nilsson, M.; Malmgren, H.; Samiotaki, M.; Kwiatkowski, M.; Chowdhary, B. P.; Landegren, U. Padlock Probes: Circularizing Oligonucleotides for Localized DNA Detection. *Science* **1994**, *265*, 2085–2088.
 - (7) Banér, J.; Nilsson, M.; Mendel-Hartvig, M.; Landegren, U. Signal Amplification of Padlock Probes by Rolling Circle Replication. *Nucleic Acids Res.* **1998**, *26*, 5073–5078.
 - (8) Nilsson, M.; Barbany, G.; Antson, D. O.; Gertow, K.; Landegren, U. Enhanced Detection and Distinction of RNA by Enzymatic Probe Ligation. *Nat. Biotechnol.* **2000**, *18*, 791–793.
 - (9) Lizardi, P. M.; Huang, X.; Zhu, Z.; Bray-Ward, P.; Thomas, D. C.; Ward, D. C. Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification. *Nat. Genet.* **1998**, *19*, 225–232.
 - (10) Liu, M.; Zhang, Q.; Chang, D.; Gu, J.; Brennan, J. D.; Li, Y. A DNAzyme Feedback Amplification Strategy for Biosensing. *Angew. Chem. Int. Ed.* **2017**, *56*, 6142–6146.
 - (11) Dahl, F.; Banér, J.; Gullberg, M.; Mendel-Hartvig, M.; Landegren, U.; Nilsson, M. Circle-to-Circle Amplification for Precise and Sensitive DNA Analysis. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 4548–4553.
 - (12) Murakami, T.; Sumaoka, J.; Komiyama, M. Sensitive Isothermal Detection of Nucleic-Acid Sequence by Primer Generation-Rolling Circle Amplification. *Nucleic Acids Res.* **2009**, *37*, e19.
 - (13) Liu, H.; Li, L.; Duan, L.; Wang, X.; Xie, Y.; Tong, L.; Wang, Q.; Tang, B. High Specific and Ultrasensitive Isothermal Detection of MicroRNA by Padlock Probe-Based Exponential Rolling Circle Amplification. *Anal. Chem.* **2013**, *85*, 7941–7947.
 - (14) Xiang, Y.; Zhu, X.; Huang, Q.; Zheng, J.; Fu, W. Real-Time Monitoring of Mycobacterium Genomic DNA with Target-Primed Rolling Circle Amplification by a Au Nanoparticle-Embedded SPR Biosensor. *Biosens. Bioelectron.* **2015**, *66*, 512–519.
 - (15) Huang, J.; Li, X. Y.; Du, Y. C.; Zhang, L. N.; Liu, K. K.; Zhu, L. N.; Kong, D. M. Sensitive Fluorescent Detection of DNA Methyltransferase Using Nicking Endonuclease-Mediated Multiple Primers-like Rolling Circle Amplification. *Biosens. Bioelectron.* **2017**, *91*, 417–423.
 - (16) Wang, Z. Y.; Li, F.; Zhang, Y.; Zhao, H.; Xu, H.; Wu, Z. S.; Lyu, J. X.; Shen, Z. F. Sensitive Detection of Cancer Gene Based on a Nicking-Mediated RCA of Circular DNA Nanomachine. *Sensor. Actuat. B Chem.* **2017**, *251*, 692–698.
 - (17) Gao, Z.; Wu, C.; Lv, S.; Wang, C.; Zhang, N.; Xiao, S.; Han, Y.; Xu, H.; Zhang, Y.; Li, F.; Lyu J.; Shen Z. Nicking-Enhanced Rolling Circle Amplification for Sensitive Fluorescent Detection of Cancer-Related MicroRNAs. *Anal. Bioanal. Chem.* **2018**, *410*, 6819–6826.
 - (18) Xu, H.; Zhang, Y.; Zhang, S.; Sun, M.; Li, W.; Jiang, Y.; Wu, Z. S. Ultrasensitive Assay Based on a Combined Cascade Amplification by Nicking-Mediated Rolling Circle Amplification and Symmetric Strand-Displacement Amplification. *Anal. Chim. Acta* **2019**, *1047*, 172–178.
 - (19) Blanco, L.; Salas, M. Characterization of a 3' → 5' Exonuclease Activity in the Phage Φ29-Encoded DNA Polymerase. *Nucleic Acids Res.* **1985**, *13*, 1239–1249.
 - (20) Geneva. *Global Tuberculosis Report 2018*; **2018**. [https://doi.org/ISBN 978 92 4 156539 4](https://doi.org/ISBN%20978%2092%204%20156539%204).
 - (21) Golichenari, B.; Velonia, K.; Nosrati, R.; Nezami, A.; Farokhi-Fard, A.; Abnous, K.; Behravan, J.; Tsatsakis, A. M. Label-Free Nano-Biosensing on the Road to Tuberculosis Detection. *Biosens. Bioelectron.* **2018**, *113*, 124–135.
 - (22) Gupta, S.; Kakkar, V. Recent Technological Advancements in Tuberculosis Diagnostics – A Review. *Biosens. Bioelectron.* **2018**, *115*, 14–29.
 - (23) Engström, A.; Morcillo, N.; Imperiale, B.; Hoffner, S. E.; Juréen, P. Detection of First- and Second-Line Drug Resistance in Mycobacterium Tuberculosis Clinical Isolates by Pyrosequencing. *J. Clin. Microbiol.* **2012**, *50*, 2026–2033.
 - (24) Minero, G. A. S.; Nogueira, C.; Rizzi, G.; Tian, B.; Fock, J.; Donolato, M.; Strömberg, M.; Hansen, M. F. Sequence-Specific Validation of LAMP Amplicons in Real-Time Optomagnetic Detection of Dengue Serotype 2 Synthetic DNA. *Analyst* **2017**, *142*, 3441–3450.
 - (25) Bejhed, R. S.; Zardán Gómez de la Torre, T.; Donolato, M.; Hansen, M. F.; Svedlindh, P.; Strömberg, M. Turn-on Optomagnetic Bacterial DNA Sequence Detection Using Volume-Amplified Magnetic Nanobeads. *Biosens. Bioelectron.* **2015**, *66*, 405–411.
 - (26) Mezger, A.; Fock, J.; Antunes, P.; Østerberg, F. W.; Boisen, A.; Nilsson, M.; Hansen, M. F.; Ahlford, A.; Donolato, M. Scalable DNA-Based Magnetic Nanoparticle Agglutination Assay

- for Bacterial Detection in Patient Samples. *ACS Nano* **2015**, *9*, 7374–7382.
- (27) Fock, J.; Parmvi, M.; Strömberg, M.; Svedlindh, P.; Donolato, M.; Hansen, M. F. Comparison of Optomagnetic and AC Susceptibility Readouts in a Magnetic Nanoparticle Agglutination Assay for Detection of C-Reactive Protein. *Biosens. Bioelectron.* **2017**, *88*, 94–100.
- (28) Fock, J.; Balceris, C.; Costo, R.; Zeng, L.; Ludwig, F.; Hansen, M. F. Field-Dependent Dynamic Responses from Dilute Magnetic Nanoparticle Dispersions. *Nanoscale* **2018**, *10*, 2052–2066.
- (29) Donolato, M.; Antunes, P.; Bejhed, R. S.; Zardán Gómez de la Torre, T.; Østerberg, F. W.; Strömberg, M.; Nilsson, M.; Strømme, M.; Svedlindh, P.; Hansen, M. F.; Vavassori, P. Novel Readout Method for Molecular Diagnostic Assays Based on Optical Measurements of Magnetic Nanobead Dynamics. *Anal. Chem.* **2015**, *87*, 1622–1629.
- (30) Tian, B.; Ma, J.; Qiu, Z.; Zardán Gómez de la Torre, T.; Donolato, M.; Hansen, M. F.; Svedlindh, P.; Strömberg, M. Optomagnetic Detection of MicroRNA Based on Duplex-Specific Nuclease-Assisted Target Recycling and Multilayer Core-Satellite Magnetic Superstructures. *ACS Nano* **2017**, *11*, 1798–1806.
- (31) Tian, B.; Han, Y.; Wetterskog, E.; Donolato, M.; Hansen, M. F.; Svedlindh, P.; Strömberg, M. MicroRNA Detection through DNAzyme-Mediated Disintegration of Magnetic Nanoparticle Assemblies. *ACS Sens.* **2018**, *3*, 1884–1891.
- (32) Baudry, J.; Rouzeau, C.; Goubault, C.; Robic, C.; Cohen-Tannoudji, L.; Koenig, A.; Bertrand, E.; Bibette, J. Acceleration of the Recognition Rate between Grafted Ligands and Receptors with Magnetic Forces. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 16076–16078.
- (33) Daynès, A.; Temurok, N.; Gineys, J. P.; Cauet, G.; Nerin, P.; Baudry, J.; Bibette, J. Fast Magnetic Field-Enhanced Linear Colloidal Agglutination Immunoassay. *Anal. Chem.* **2015**, *87*, 7583–7587.
- (34) Zhu, G.; Hu, R.; Zhao, Z.; Chen, Z.; Zhang, X.; Tan, W. Noncanonical Self-Assembly of Multifunctional DNA Nanoflowers for Biomedical Applications. *J. Am. Chem. Soc.* **2013**, *135*, 16438–16445.
- (35) Tian, B.; Liao, X.; Svedlindh, P.; Strömberg, M.; Wetterskog, E. Ferromagnetic Resonance Biosensor for Homogeneous and Volumetric Detection of DNA. *ACS Sens.* **2018**, *3*, 1093–1101.
- (36) Østerberg, F. W.; Rizzi, G.; Donolato, M.; Bejhed, R. S.; Mezger, A.; Strömberg, M.; Nilsson, M.; Strømme, M.; Svedlindh, P.; Hansen, M. F. On-Chip Detection of Rolling Circle Amplified DNA Molecules from *Bacillus Globigii* Spores and *Vibrio Cholerae*. *Small* **2014**, *10*, 2877–2882.
- (37) Tian, B.; Han, Y.; Fock, J.; Stro, M.; Leifer, K.; Hansen, M. F. Self-Assembled Magnetic Nanoparticle–Graphene Oxide Nanotag for Optomagnetic Detection of DNA. *ACS Appl. Nano Mater.* **2019**, *2*, 1683–1690.
- (38) Strömberg, M.; Göransson, J.; Gunnarsson, K.; Nilsson, M.; Svedlindh, P.; Strømme, M. Sensitive Molecular Diagnostics Using Volume-Amplified Magnetic Nanobeads. *Nano Lett.* **2008**, *8*, 816–821.
- (39) Zardán Gómez de la Torre, T.; Mezger, A.; Herthnek, D.; Johansson, C.; Svedlindh, P.; Nilsson, M.; Strømme, M. Detection of Rolling Circle Amplified DNA Molecules Using Probe-Tagged Magnetic Nanobeads in a Portable AC Susceptometer. *Biosens. Bioelectron.* **2011**, *29*, 195–199.
- (40) Liu, M.; Zhang, Q.; Li, Z.; Gu, J.; Brennan, J. D.; Li, Y. Programming a Topologically Constrained DNA Nanostructure into a Sensor. *Nat. Commun.* **2016**, *7*, 12074.
- (41) Al-Soud, W. A.; Rådström, P. Effects of Amplification Facilitators on Diagnostic PCR in the Presence of Blood, Feces, and Meat. *J. Clin. Microbiol.* **2000**, *38*, 4463–4470.
- (42) Schrader, C.; Schielke, A.; Ellerbroek, L.; Johne, R. PCR Inhibitors - Occurrence, Properties and Removal. *J. Appl. Microbiol.* **2012**, *113*, 1014–1026.



TOC