Sequence-specific validation of LAMP amplicons in real-time optomagnetic detection of Dengue serotype 2 synthetic DNA

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Electronic Supplementary Information

S1 Optomagnetic setup and chip design

Fig. S1: Setup for real-time optomagnetic studies where four experiments can run simultaneously (only one shown). (A) The picture shows a plastic chip placed inside a setup with the top heater in the open position. (B) Schematic of each of the four setups that consists of a light-emitting diode (LED), photodetector and two coils. The LED emits monochromatic light, which is guided to the side of a chip using a plastic rod. The light transmitted through the chip is collected and guided to a photodetector using a second plastic rod. The plastic chip is sandwiched between two resistive heaters with a temperature measured by a platinum thermometer. The magnetic field on the chip is provided by an electromagnet placed on either side of the chip. C. Photo showing dimensions of plastic chip.

The optomagnetic setup consisted of four duplicates of a single-chip setup such that four experiments could run in parallel. Each single-chip setup measured the transmission modulation of monochromatic light ($\lambda = 470$ nm) through a suspension of magnetic nanoparticles (MNPs) when an external oscillating magnetic field was applied to align the particle magnetic moments. Two coils supplied by a custom-built voltage-to-current converter (shared between all four setups) generated a sinusoidal homogenous magnetic field. Optomagnetic spectra were measured at field amplitude 0.8 mT and comprised 41 logarithmically spaced frequencies between $f = 1$ and 2800 Hz, a spectrum was measured every 40 s. The light from a blue LED was guided in a $\varnothing 5$ mm PMMA rod to and from the sample chamber, see Fig. S1A. The light was detected with a photodetector.
(PDA36A, Thorlabs) connected to a data acquisition unit (USB-6341 DAQ, National Instruments). The DAQ measured the signals from the photodetectors from all four setups simultaneously. Each sample chip was fabricated by laser cutting and bonding with pressure sensitive adhesive three layers of PMMA (total thickness ca. 3 mm, width 13 mm, length 25 mm). The measurement chamber containing the sample had square geometry and an inner volume of 75 µl, see Fig. S1C. In each setup, the chip was sandwiched between two custom-made resistive heater elements (=2.5 Ω) defined by photolithography on a 1.5 mm thick aluminium printed circuit board. The temperature of each chip was monitored using a Pt100 thermometer and regulated by means of a four-channel Stanford Research Systems PCT10 temperature controller.

In our present setup we chose for simplicity and reliability to use state-of-the-art off-the-shelf components for the setup costing an estimated total of about 9 k€ for the setup for simultaneous measurements on four chips, excluding mechanical components and labour. In a production setting, we believe that this cost can be significantly reduced by use of components tailored to the application to replace the data acquisition card (1.200 €), the temperature control electronics (5.000 €) and the photodetectors (300€ each). The chip disposable in the present study is a simple transparent plastic container, which can be made at very low cost by injection moulding in a mass production setting.

**S2. Target and primers for LAMP**

Primer design for loop-mediated isothermal amplification (LAMP) of Dengue synthetic target DNA (D2) was adapted from Lau et al.² (Table S1). Sets of four to six primers were used to generate D2 amplicons from 3’-non-coding region (nucleotide sequence frame 10477-10685 out of the complete genome taken from NCBI database, strain BR64022). Bst 3.0 polymerase possesses reverse transcriptase activity and, therefore, is also capable of RNA amplification. However, we have chosen synthetic target to decrease complexity of handling the LAMP cocktail.

Synthetic target containing Dengue sequence TACGCATGCGTAGTGGACTAGCGGTTAGAGGAGACCCCTC... was ordered from IDT (Integrated DNA Technologies) as gblock.

| Table S1. DNA sequences of the LAMP primers given in the 5’→3’ direction. |
|-------------------|-------------------|-------------------|-------------------|
| FIP               | TCATCTCACCTTGGCCCCCGTGGATAGGAGACCCTCTC |
| BIP               | AGAGGTTAGAGGAGACCCCTTTGTTGCTGGGATTTTG |
| F3                | TACGCATGCGTAGTGGAG | B3                | GCGTTCTGTGCTGGAAAT |
| LF                | GTTGTGGCGATTTGTAAGG |
| LB                | ACAGCATATTTGACGGCTTTG |
| LV                | ATTTGGTGGCGATTTGTAAGG |

According to previous reports on LAMP, outer forward (F3) and backward (B3) primers are used only in the very early stage of LAMP. They are required to displace DNA copies generated by extension of forward inner
(FIP) and backward inner (BIP) primers that bind to their targets downstream binding sites of F3 and B3, respectively. This leads to two dumbbell structures made from target DNA, which can bind two primers each via their loops: FIP and LB or BIP and LF (Fig. 1). The reverse complementary sequences (cFIP, cLB, cBIP, cLF) are incorporated into LAMP products in periodic order.³ Amplicons continuously undergo transformations of priming → strand displacement → self-priming → strand displacement → priming etc. (Fig. 1), which leads not only to amplification, but also to elongation of amplicons as discussed in the main manuscript.

Multiple freezing/thawing of primers could lead to formation of primer homodimers. This was avoided by distributing primers into small aliquots. All DNA primer sequences were ordered from DNA Technologies with standard purification. The biotinylated sequences FIP and LV were ordered with HPLC purification grade. Only one of the primers/probes in LAMP cocktail was biotinylated for studies of LAMP on MNPs. Biotinylation of FIP and LF at the 5'-end was used to study elongation of the anchored amplicons via probing hydrodynamic size of the functionalised MNPs. Biotinylation of LV at the 3'-end was used to discriminate true positive and spurious amplicons via probing amplicon’s sequence in the elongation phase. In this case, MNP attached to the 3'-end was blocking the access of Bst polymerase and, therefore, preventing extension of LV.

Nucleotide sequence alignment (BLAST, https://blast.ncbi.nlm.nih.gov) of the LV-probe sequence with the whole genomes of the four Dengue stereotypes revealed following max possible length of intact duplex without gaps: 9 bp (D1), 22 bp (D2), 9 bp (D3), 8 bp (D4). Therefore, the LV-probe should be also able to discriminate D2 target from other serotypes in a sequence-specific manner. We also would like to exemplify similarity of the 3'-NCR sequences between D2 (in bold) and D3. The sequence targeted with LV-probe is highlighted in green.

S3. Detection approach I: Real-time LAMP

Figure 1 in the main manuscript shows a schematic illustration of the LAMP reaction that leads to an increase of the hydrodynamic volume of MNPs. We demonstrate real-time detection of Dengue amplicons through monitoring of the hydrodynamic volume of MNPs in the optomagnetic signal. For this, the FIP primer (Table S1) was biotinylated, and the mechanistics of LAMP was studied on MNPs. Accumulation of spurious amplicons in the lab environment was also traced in real-time while running LAMP on a dilution series of D2 (Fig. S2). We
came to a conclusion that the concentration of spurious amplicons in NTC was on the order of 100 fM after 9 days of running LAMP, compare violet plot (100 fM D2 in “clean” conditions) in Fig S2A to blue plot (NTC, day 9) in Fig. S2B.

**Fig. S2:** Studies of real-time LAMP with detection approach I. (A) Optomagnetic detection of Dengue amplicons (violet) originating in LAMP from 100 fM D2 as well as from NTC (blue). (B) Accumulation of spurious amplicons seen by a trend of threshold times that decrease with the number of days during which LAMP experiments were carried out.

Amplicons undergo similar changes led by “annealing” and extension of biotinylated LF. Better discrimination was achieved with LF probably because of (1) its shorter length (less probability of forming homodimers) and (2) competing extension mediated by self-annealed 3’-end upstream the LF.
S4. Detection approach II: DNA melting

The sequence of amplicons was further studied using DNA melting. In this technique, the gradually increasing temperature allowed us to study the equilibrium between bound and unbound DNA strands. LAMP amplicons were captured on functionalised MNPs via DNA complex of loop validating (LV) probe and the loop, i.e. LV: loop. Binding between the two DNA strands was fast and led to a change in hydrodynamic volume of the MNPs corresponding to ca. 10 Hz (at 30°C). Upon increasing temperature, unwinding of the strands led to detachment of the large amplicons from the MNP surface with a sharp melting transition (Fig. 4). Fig S3A displays a series of second harmonic optomagnetic spectra for one of the melting studies in real-time. Signal intensity stayed unchanged unlike in the real-time LAMP (Fig. 2A). This suggests that there was no loss of MNPs, at least during the melting studies, whereas in real-time LAMP studies a drop of the optomagnetic signal was associated with formation of magnesium pyrophosphate and following electrostatic interactions with amplicons on MNPs.

Fig. S3: Sequence-specific discrimination of amplicons on capture probes via DNA melting. (A) Series of real-time optomagnetic spectra recorded during melting of spurious amplicons captured on MNPs (0.01 °C/s), one spectrum recorded every 40 s. The arrow indicates the time progression. (B)-(D) End-point detection of true positive (red) and spurious (blue) amplicons captured on MNPs via attached DNA LV, obtained at 67°C. Amplicons were produced from samples containing 1 pM of target (red) and no target (blue), respectively: (B) no looped primers, 80 min LAMP, (C) LB – 0.4 µM, 60 min LAMP, and (D) LB – 0.2 µM, 80 min LAMP. After an inactivation step (see Materials and Methods), amplicons were incubated with LV- functionalized MNPs.

It is important to notice that the looped primers are used only to accelerate LAMP. We eliminated the LF primer from the system (otherwise it would compete with LV), and varied the concentration of LB (Fig. S3). Without both looped primers LF and LB, the degree of discrimination was still low due to insufficient
amplification rate (Fig. S3B). Increasing the concentration of LB to 0.4 µM accelerated the amplification of both amplicons and also resulted in a poor discrimination of amplicons at 67°C (Fig. S3C). We considered 0.2 µM LB as the optimum concentration for discrimination of the amplicons (Fig. S3D).

S5. LAMP-optomagnetic detection of DNA nanoclusters
A difference in $T_m$ of more than 20°C (Section S4) allowed us to discriminate between true and false-positive signals at 67°C (the LAMP operating temperature). As mentioned in Section S2, reverse complementary sequences $cFIP$, $cLB$, $cBIP$, $cLF$ are incorporated into LAMP products in a periodic order. In the elongation phase, a single stranded tract containing the sequence repeats is exposed and used as a target for LV-MNPs, where LV is 3'-biotinylated analogue sequence of LF. Multiple binding of the biotinylated LV probe on single stranded amplicons caused reproducible clustering of the MNPs, which was clearly detectable in optomagnetic signals $V_2'$ and $V_2''$ (Fig. S4). We speculate that amplification of spurious amplicons competes with amplification of target for dNTPs and lowers the LOD. For example, if there is 100 fM of spurious amplicons and 10 fM target, amplification of target “starts” when spurious amplicons are finishing using up reagents for their own amplification. For this reason, spurious amplification is often called parasitic amplification.

The optomagnetic spectra display depletion of single MNPs to different extent depending on the presence of target in the LAMP mixture (Fig. S4). The depletion of single MNPs is accomplished by formation of larger clusters clearly detectable in the optomagnetic signal by the signal sign inversion at lower frequencies (1-20 Hz)$^4$. Minor depletion of MNPs takes place also for the NTC (Fig. S4C). We speculate that this minor depletion of MNPs in the NTC is caused by attaching capture oligonucleotides to magnesium pyrophosphate and subsequent sedimentation of the MNPs without clustering, which was also observed in detection approach I (see Fig. 2A in the main text). At the end of experiment, we observed the orange precipitate with fraction of MNPs sedimenting at the bottom of the chip.
**Fig. S4:** Real (top) and imaginary (bottom) optomagnetic spectra recorded in real-time with LV-MNPs during LAMP amplification of (A) 1 pM D2 target, (B) 300 fM D2 target, and (C) NTC. The spectra were obtained in real-time every 40 s in the order red→orange→yellow→green→cyan→blue→violet. Clustering is observed as a signal at low frequencies. (D) Mechanism of MNP clustering as response to amplification of binding sites on a single amplicon in the elongation phase. To perform the reaction, 65 µl of the “asymmetric” LAMP mixture was mixed with 5 µl of LV-functionalised MNP solution and 30 µl aliquots of the D2 target made by serial dilution from the 10 nM stock solution. The end concentration of MNPs was 0.05 mg/ml or ca. 50 pM, and the MNP surface was pre-saturated by LV-probe (>100 probes/MNP).

We also observed similar trend of clustering clearly detectable in optomagnetic signal in the systems containing 5'-biotinylated LF primers instead of 3'-biotinylated LV (of the equal concentration), see **Fig. S5**. In this case, there were no MNP-free LF primers in the system, which would compete for binding to the amplicons in the elongation phase of LAMP. Clusters of MNPs were formed only in the presence of the target DNA, which generated binding sites in true-positive amplicons for the MNP-bound capture probes. However, the 3’-end of the LF was active in the elongation phase, which, in contrast to **Fig. S4**, led to a peak shift (i.e. like in the detection approach I, **Section S3**) in both true positive as well as false positive LAMP reactions **Fig. S5B**. In the target-specific LAMP, clustering was predominant, although with a peak shift observed in early stage. In course of time, MNPs were displaced (**Fig. S5D**) with DNA amplicons attached ($f_{\text{peak}}$ ca. 50 Hz).
Fig. S5: Optomagnetic detection of LF-MNPs binding to amplicons during LAMP. (A)-(B) Spectra recorded in real-time (dark to light colour) during amplification of (A) NTC (contaminated) and (B) 300 fM D2 target. (C) Values of $f_{\text{peak}}$ vs. time representing LAMP in real-time obtained from the data in (A) (black) and (B) (green circles). The almost complete depletion of MNPs in (B) resulted in noisy values of $f_{\text{peak}}$ after $t \approx 40$ min (not shown). (D) Depletion of MNPs followed by turn-on signal due to MNP displacement (green→light green→cyan→blue). LAMP settings were similar to the one exemplified in Fig. S4 except of MNPs functionalised with 5’-biotinylated LF primer instead of 3’-biotinylated LV-probe.

References