Electronic Supplementary Material (ESI) for Analyst.

Real Time Plasmonic qPCR: How fast is Ultra-fast? 30 cycles in 54 seconds.

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Supplemental Material

S1. Master Mix Preparation and Master Mix study

Stock Nanoparticle PEGylation

Using UV/VIS spectroscopy it is possible to determine a highly consistent stock solution that is added to each master after dilution to a standard absorbance of 0.5 (optical density) on a nanodrop spectrometer. PEGylation was performed over a 24 hour period. Ten samples (500µL) of Stock nanorods (0.4mM) were mixed with 500µl of HS-PEG5000 (10mg/mL) and vortexed. Tube were left at room temperature for 24hrs, producing a small color change to darker red (scattering from transverse peak denotes visible color). Purification was achieved by centrifugation at 12,000 rpm for 20 minutes and 3 wash steps using dionized sterile water. Final resuspension volume was varied dependent upon the stock concentration desired in the 10nM to 50nM range, with nanoparticles stored at 4^{o}C

Primer sequenced used in Plasmonic PCR experiments

Primers used in Figure 2A:

Gene specific primers of Thyroglobulin segment cloned into TOPO-TA vector:

Forward: 5'-GTACTAGTGGGACATTTCC-3';

Reverse: 5'-AAAAGCGGCGTCTCTGATAG-3'

Product: 900 bp

Primers used in Figure 3A:

Human AR gene inserted into BHEX plasmid:

Forward: 5'-GCTCCCACTTCCTCCAAGGACAATTAC-3';

Reverse : 5'-CTGCCTTACACAACTCCTTGGC-3'

Product: 79bp

Primers used in Figure 2B

Human Glucokinase A457 clone in pTwin2 vector

Forward:

5’-GCCCTGGTCTCGGCGGCCTGTAAGAAGGCCTGCGTATCCGGTGACACCATT-3’;

Reverse: 5’-GCTGCAATGATACCGCGAGACCC-3’

Product size: 120bp

Primers used in Figure 2C and Figure S4-1:

Human AR gene inserted into BHEX plasmid:

Forward: 5’-AACCAGCCCGACTCCTTTG-3’

Reverse: 5’-CAGGGGCCAAGTAGAGCATC 3’

Product size: 274bp

Primers used in Figure S4-2

Human Glucokinase A457 clone in pPtwin2 vector:

Forward: 5’-GGTGGTCATATGGCATGGATGTCACAAGGAGCC-3’;

Reverse: 5’-GCGCGTAGACACGCTCTCGCAGGCG-3’

Product size: ~ 1000 bp

Primers used in Figure S4-3

Human Glucokinase A457 clone in pPtwin2 Vector:

Forward: 5'-GCGTGTCTACGCGCGCTGCGCSCSTGTGC-3';

Reverse: 5'-GGTGGTTGCTCTTCCGCAGGCCTTCTTACAGGCCACCGCC-3'

Product size: ~500bp

Primers used in Figure S4-4 and S4-5

EGFR from cDNA prepared from Invitrogen Superscript reverse transcriptase kit using MCF7 cell extract. 100bp product from exon 18-21:

Forward: 5'-AGGATCTTGAAGGAAACTGA-3';

Reverse: 5'-GTAGGCTTCATCGAGGATT-3'

Master Mix optimization and enzyme testing

As a refinement of our PCR method, the PEGylation of the gold nanorods (referred to also as gold colloid) was an improvement such that the large nanoparticles would not interfere with the function of the polymerase. Using a both a conventional Mastercycle PCR machine and our plasmonic PCR platform and increasing concentration in the gold colloid mixes for different enzymes, it became possible to completely replace the water fraction with the PEG-functionalized gold nanorod colloidal solution and still have no inhibitory effect upon amplicon production (**Table S1-1**). Each gel shown below contains a serial dilution of the gold colloid solution. A final important note of the dilution series of the gold colloid solution, is that the negative plasmonic PCR (-vp), proves that the colloid solution alone does not contribute to contamination during the PCR reaction.

Table S1-1: Volume Ratios of H_2O to Gold colloid nanoparticle preparation used in plasmonic PCRs.

	H_2O (µL)	Colloid (µL)
- ve (negative conventional PCR)	19.9	0
+ve (positive conventional PCR)	19.9	0
- vp (negative plasmonic PCR)	6	13.9
Plasmonic PCR with increasing concentrations of colloid		
P1	19.9	0
P2	13.9	6
P3	10.9	9
P4	7.9	12
P5	3.9	16

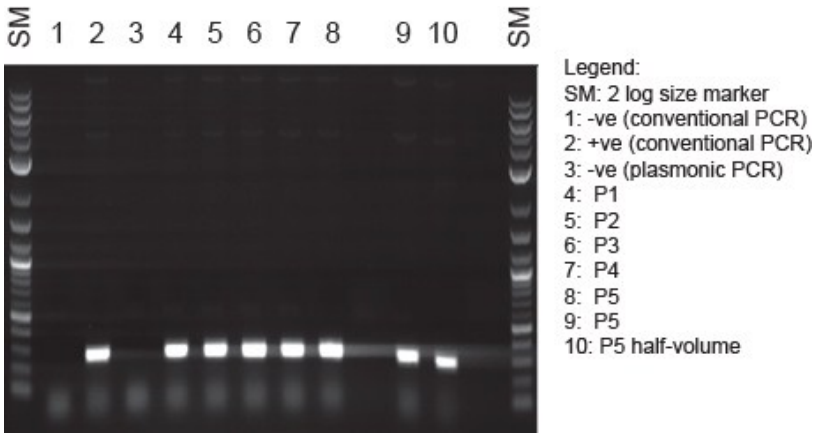

Figure S1-1: Comparison of DNA amplification with increasing concentrations of gold colloid. Template is plasmid DNA and product is 274bp. Increasing gold colloid in reaction mix through lanes 4-8, lane 9 uses a lower template concentration and lane 10 uses a half loading of DNA mix. Lanes 8, 9 & 10 completely replace the water fraction of the PCR with colloid.

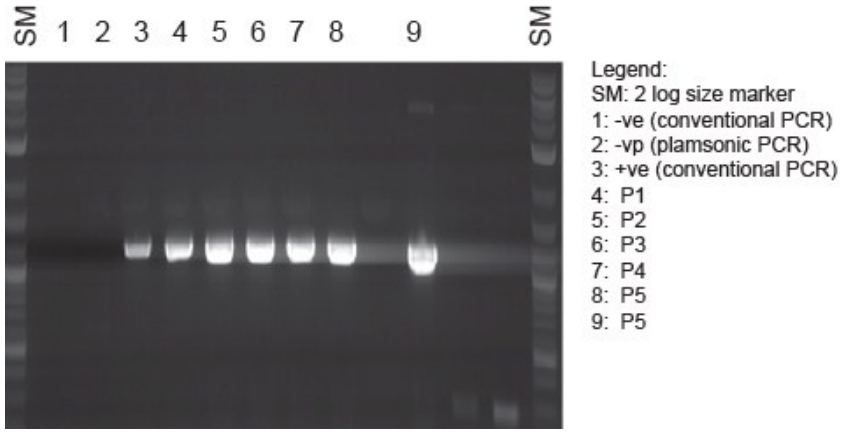

Figure S1-2. Plasmonic PCR amplification of 1Kb DNA fragment. For longer fragments (also in the case of Figure S4-3) increasing nanoparticle concentration significantly improves the yield for the PCR reaction.

The pattern is replicated with a PCR performed upon human genomic DNA for the androgen receptor soon in Figure S4-3. The enzyme used in both figure S4-2 and S4-3 is Phusion polymerase.

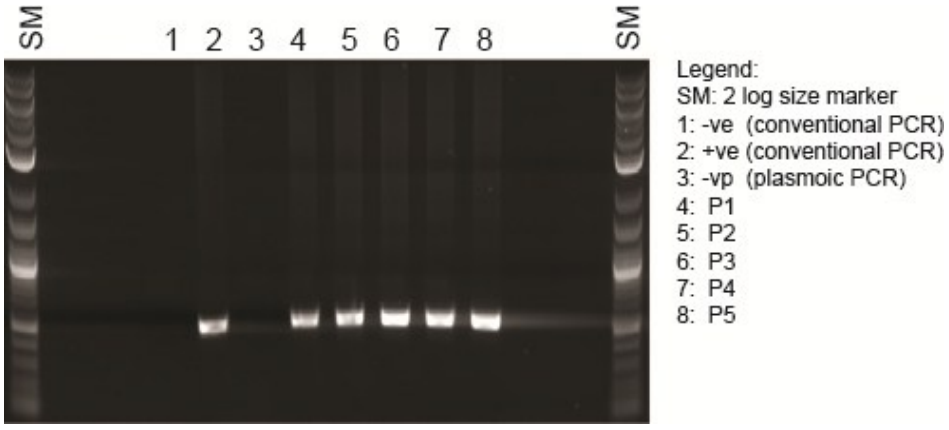

Figure S1-3. Genomic DNA amplification fragment size of 500bp.

Figures S1-4 and **S1-5** considers the use of a single stranded cDNA template generated by reverse transcriptase. Presence of nanoparticles does not adversely affect product generation with either Phusion or LA Taq polymerase enzymes.

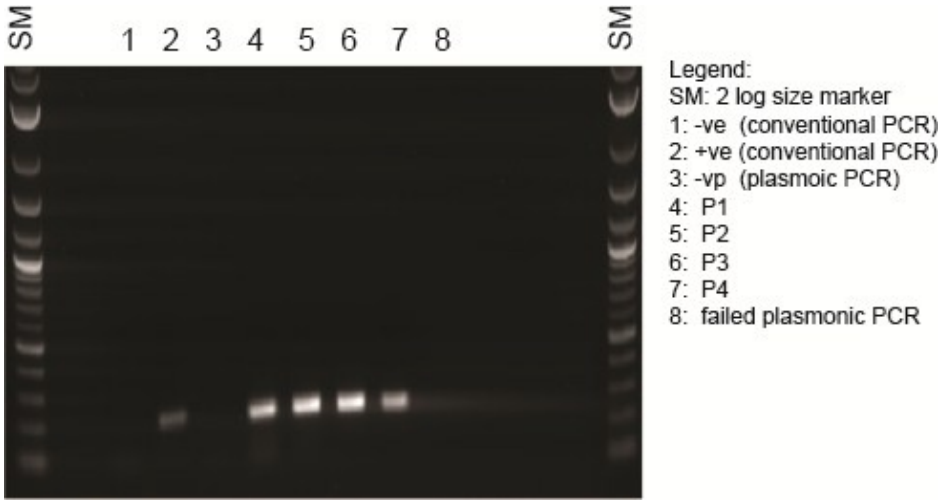

Figure S1-4. Phusion polymerase amplification of EGFR cDNA from MCF-7 breast cancer cell lines. Source of ssDNA is reverse transcription of mRNA from MCF-7 breast cancer cell line.

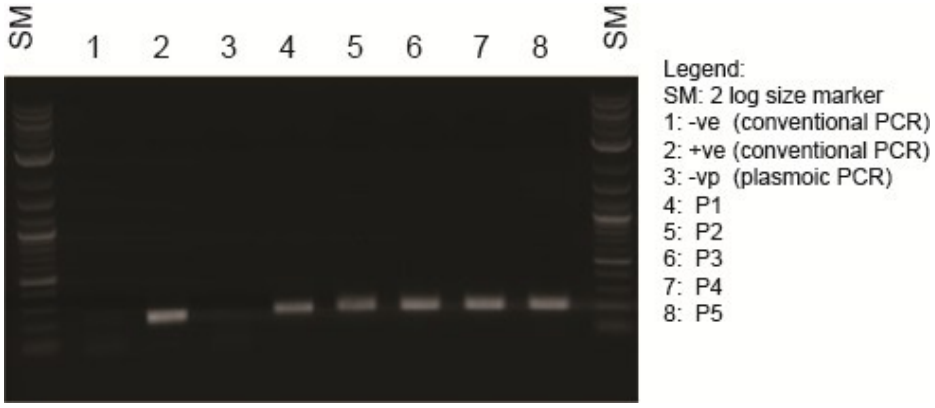

Figure S1-5. LA Taq amplification of EGFR cDNA from MCF-7 breast cancer cell lines. (fragment 120-130bp). To study if this effect perpetuated with smaller amplicons, a PCR reaction for a segment of the EGFR mRNA was performed. Phusion polymerase (Figure S4-4)

shows increasing band intensity versus the non-colloid positive control an indication of increase in PCR yield. However, LATAQ polymerase demonstrates consistent band intensity (lanes 4-8). This is explained by LA Taq polymerase having a lower processivity than Phusion polymerease; as LA Taq is designed specifically for high molecular weight amplifications, so yield changes due to increasing nanoparticles do not appear to yield any amplicon difference.

PCR Mixes

Prior to use in PCR, expose plasmonic colloid and plasticware to UV light for 2hrs before inclusion in any PCR reactions to reduce contamination and vortex before use. Storage between use at 4^{o}C and is prepared in dionized sterile water. The following examples show how master mixes can be altered using the substitution of the water fraction for plasmonic colloid.

For amplification from plasmid DNA of 273bp and 1kb product sizes, Figure S4-1 and S4-2, respectively. Water fraction can be substituted for plasmonic colloid as part of a serial dilution or as a total water replacement.

Components/PCR reaction	**Volume (µL)**
5 X Phusion HF Reaction Buffer (NEB)	6.0
dNTPs (10 mM) (NEB)	0.6
Primer 1 (10 pmol/µL)	1.5
Primer 2 (10 pmol/µL)	1.5
DNA* or d_2H_2O for –ve controls	1.0
Purified BSA (100X)	0.3
Phusion High Fidelity DNA polymerase (2U/µL)	0.3
d_2H_2O	19.9
Total	31.1

For LA TAQ genomic PCR plasmonic mix can be substituted for water fraction as part of serial dilution or as total water replacement. Adaptation of protocol was used for LA Taq EGFR cDNA amplification (120-130bp).

Components/PCR reaction	**Volume (µL)**
10 X LA Taq Buffer II (Mg^{2+} plus) (TaKaRa)	5.0
dNTP mixture (2.5 mM) (TaKaRa)	8.0
Primer 1 (25 pmol/µL)	0.5
Primer 2(25 pmol/µL)	0.5
gDNA (320 ng/µL) or d_2H_2O for –ve controls	3.0
Purified BSA (100X)	0.2
LA Taq DNA polymerase (5U/µL)	0.5
d_2H_2O	32.5
Total	50.2

PCR mix for Phusion cDNA as used for EGFR amplification (fragment 120-130 bp)

Components/PCR reaction	**Volume (µL)**
5 X Phusion HF Reaction Buffer (NEB)	4.0
dNTPs (10 mM) (NEB)	0.4
Primer 1 (25 µM)	0.4
Primer 2 (25 µM)	0.4
DNA or d_2H_2O for –ve controls	1.0
Purified BSA (100X)	0.2
Phusion High Fidelity DNA polymerase (2U/µL)	0.2
d_2H_2O	13.6
Total	20.2

For more efficient photothermal conversion the laser focus can be increased, hence increasing heating rate, or increasing the concentration of gold nanorods in the beam path. Both methods have been found to be cost-effective and efficient in generating controllable heating ramps between 40-70°C/s.

KAPA2G fast Master Mix

Kapa2G master mix preparation was performed as for Phusion and LATAQ polymerases by substitution of the water fraction for plasmonic mix.

Components/PCR reaction	**Volume (µL)**
PCR DNase free water or colloid gold	15.9
5X KAPA2G buffer	5
10mM dNTP mix	0.5
10µM Forward Primer	1.25
10µM Reverse Primer	1.25
Template DNA	1
5 units KAPA2G fast DNA polymerase	0.1
Total	25

Standard temperature conditions are: Hot start (95°C, 3 minutes), denaturation (95°C, 15s), annealing (60°C, 15 seconds), elongation (72°C, 1s). KAPA2G fast works with much shorter hot start/denaturation and annealing times, to enable ultrafast cycling. Of available enzymes it has the most favorable processivity at 1kb/s, that when attempting amplification of less than 100bp requires the maintenance of temperature for extension. In ultrafast PCR the yield is solely limited by the processivity of the enzyme.

Plasmonic Emulsion PCR

For the performance of a reverse phase plasmonic emulsion PCR primers for androgen receptor were used (Lenore insert primer sequences) and Human genomic DNA was used as the template using same conditions as listed above for LA Taq. A 4X PCR master mix was prepared (4 x 25µL or 2 x 50µL in the case of LA Taq. Using 50µL of emulsion oil from kit, the master mix was added and vortex to establish the emulsions. 25µL of the emulsion was aliquoted to the glass tube and placed in the PCR instrument. A convention stepwise PCR was performed with Denaturation (98°C, 10s), annealing (50°C, 10s) and extension (72°C, 15s).

S2. Plasmonic PCR Thermocycling

Time	Laser Stability	Temperature	Lock-in Amplifier	Cycle
31.90	25.25	69.61	287	10
31.93	25.21	69.61	289	10
31.96	25.15	69.34	287	10
31.99	25.25	69.26	284	10
32.02	25.25	69.26	297	10
32.05	25.25	75.25	284	10
32.07	25.25	75.25	290	10
32.10	25.25	75.25	285	11
32.13	25.25	75.25	287	11
32.16	25.25	77.02	284	11
32.19	25.15	77.02	285	11
32.22	25.21	77.02	285	11
32.24	25.21	78.04	283	11
32.27	25.22	78.04	285	11
32.30	25.25	78.04	279	11
32.33	25.25	78.04	287	11
32.36	25.25	81.88	279	11
32.38	25.25	81.69	285	11
32.41	25.25	81.69	278	11
32.44	25.25	84.67	279	11
32.47	25.22	84.67	281	11
32.50	25.28	84.67	279	11
32.52	25.25	84.67	286	11
32.55	25.25	86.80	275	11
32.52	25.25	86.80	286	11
32.61	25.25	86.80	279	11
32.63	25.21	88.54	285	11
32.61	25.25	90.54	282	11
32.69	25.25	90.54	280	11
32.72	25.26	90.25	283	11
32.75	25.21	90.25	282	11

Figure S2-1. Screen Capture of Plasmonic PCR Cycling video, demonstrating rapid thermocycling, between 93°C and 68°C, over 30 cycles using the plasmonic PCR. Here the elongation and annealing temperatures are the same (68°C). **Video S1** starts at about the 5th cycle and proceeds and terminates at the 30 cycle.

S3. Thermal Imaging

At various stages the consideration of the thermal distribution within the tube needs to be raised. From our observations using a thermal imager we were able to confirm even heat distribution throughout the 25μL volume for the PCR, while simultaneously referencing to the conventional calibrated MLX90416 IR thermopile. **Video S2**, is a confirmation of the thermal imaging (left) and thermopile (right) monitoring of temperature within the tub. Both methods are self-calibrating with internal sensors. Therefore, the thermal conductivity through the tube, with both the thermopile and thermal imaging, are reasonably accurate.

S4. Plasmonic Measurement

The lock-in amplifier was tested by altering the plasmonic resonance of the gold nanoparticles. In principle, as DNA concentration increase as a result of amplification and the proximity of the amplicons with the nanoparticles, the resonance will shift to longer wavelengths, shown in Figure 4 and Figure 5. The advantage of probing at the minima between the resonances is the greatest change in absorbance/transmission can be achieved in this position. It is analogous to the measurement technique in conventional fixed angle surface plasmon resonance. Conversely the broader 808nm resonance can move around the 808nm laser light without adversely affecting the heating rate of the PCR system.

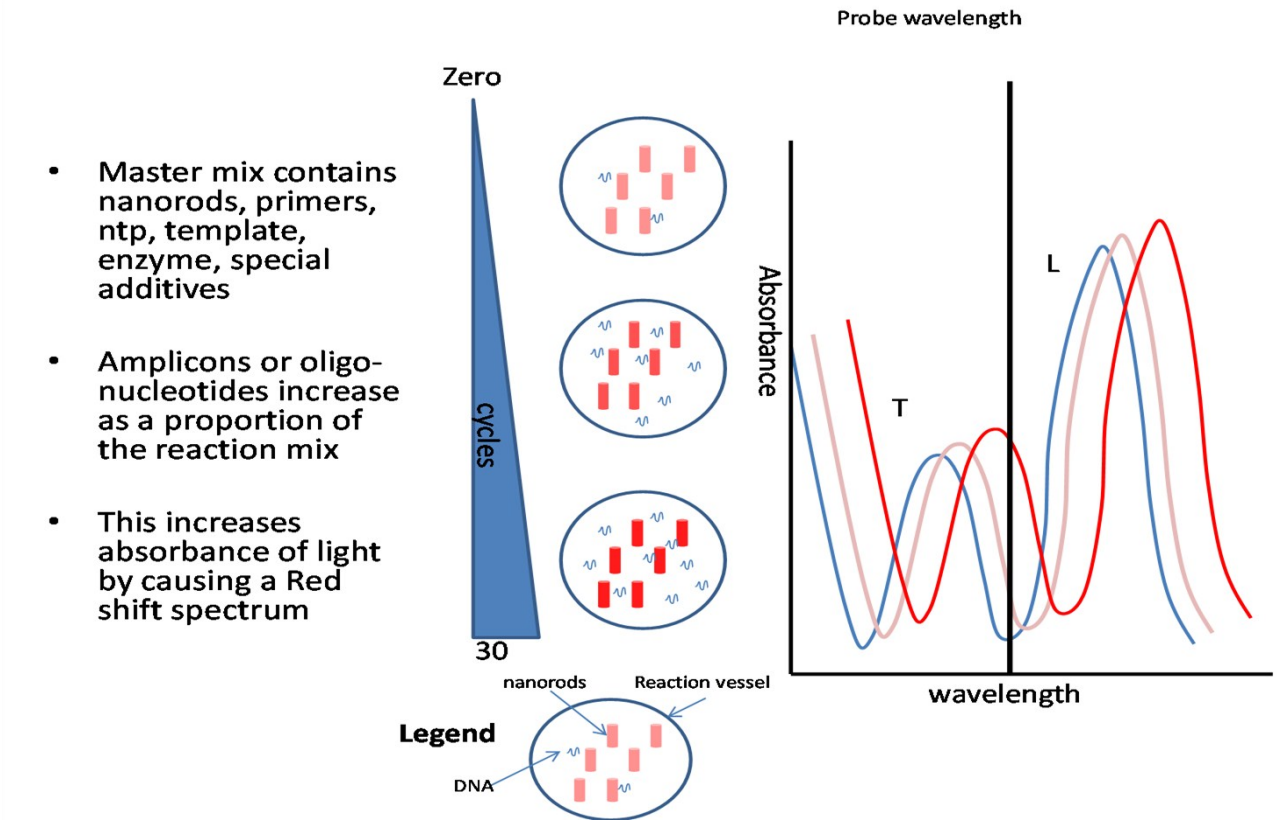

Figure S6-1.General Example of plasmonic measurement of increasing amplicons generation in PCR.

S5. Video Stills and Graph for Fluorescence

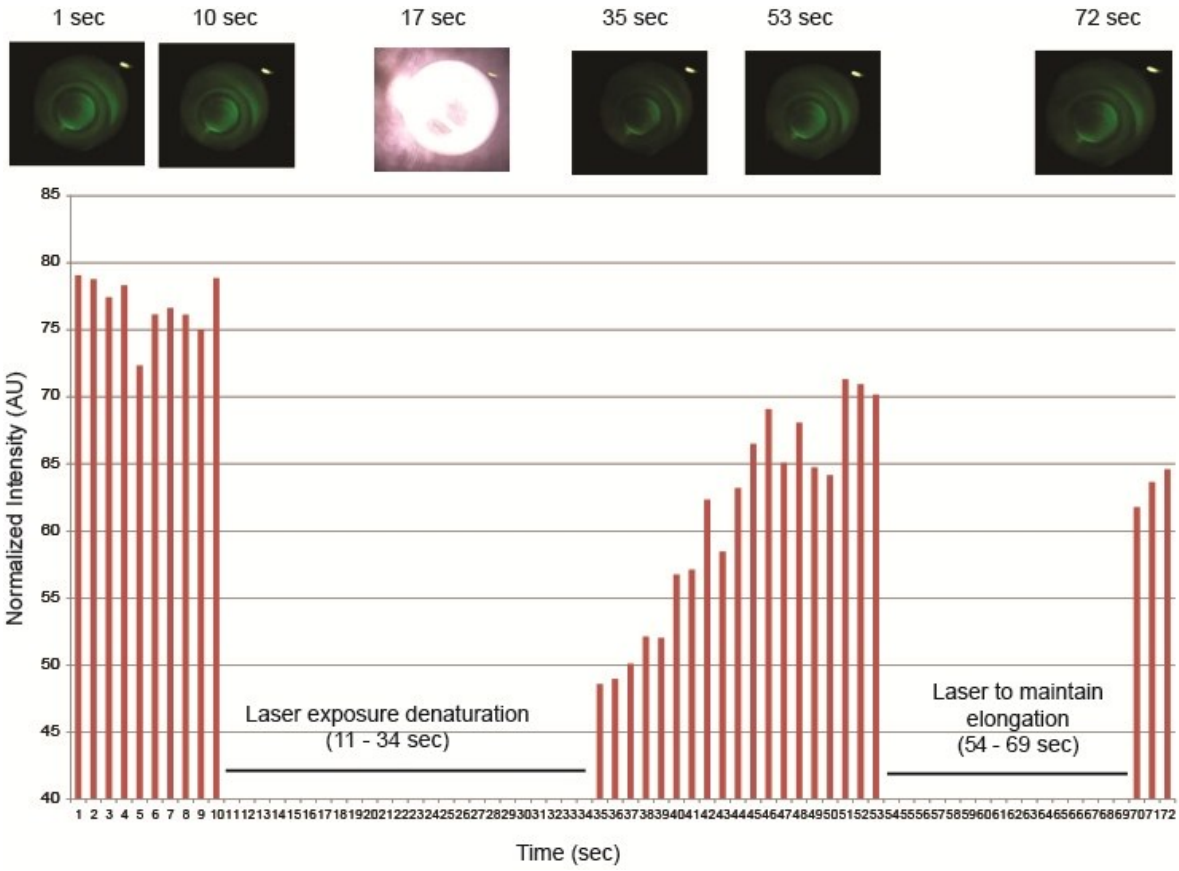

Figure S5-1. To validate the potential use for fluorescence integration in real time with plasmonic PCR, the process was visualized using a Raspberry Pi camera with a filter matching the FITC bandwidth. The filter allows the pass-through of the heating laser scattering, with the consideration of the TTL modulation. The experiment was set up to investigate whether fluorescence can be measured in conjunction with the cycling required for plasmonic PCR. Using only genomic DNA (100 ng/µL) and picogreen (2 µL for 25 µL of reaction), the experiments demonstrates that fluorescence disappears during the heating phase and recovers during cooling & annealing phases of the PCR cycle. See **VideoS3**.

S6. LAMP with Plasmonic PCR system

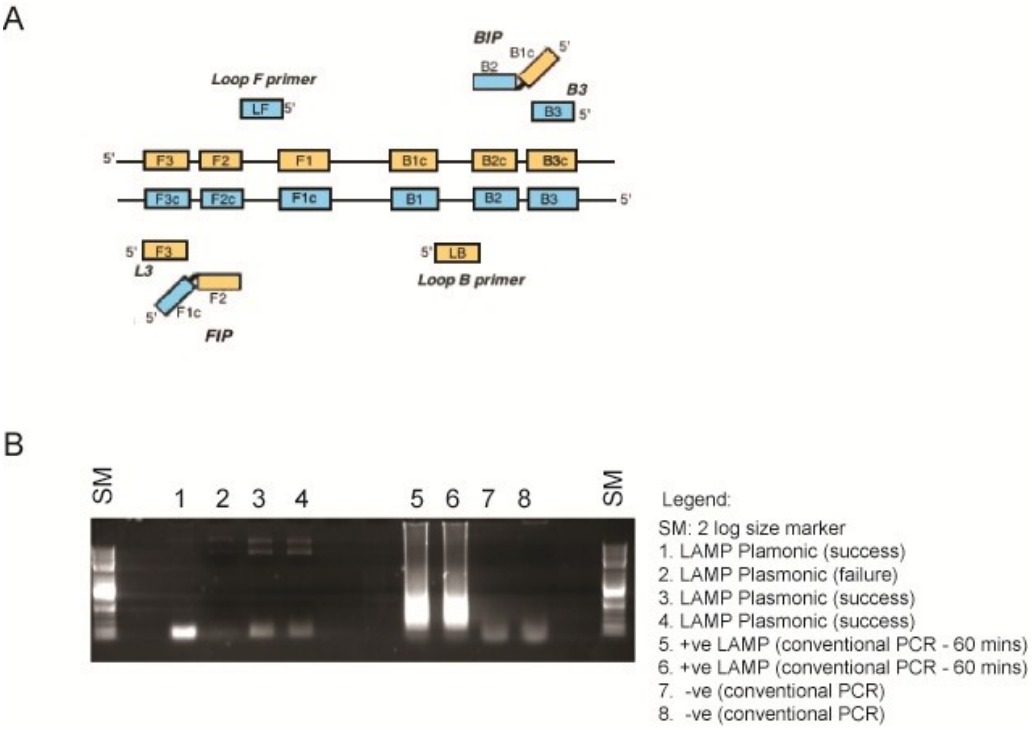

Figure S6-1. To demonstrate that other DNA amplification methodologies can be applied, a Loop mediated isothermal DNA amplification (LAMP) approach was demonstrated. In a previous work (see Carnevale *et al., Anal. Methods*, 2015,**7**, 287-295)[1] our group developed a *E.coli* serogroup identification technique. **A.** Schematic of LAMP primers with the complementary regions. **B**. Using the LAMP primer sets for general *E.coli* identification comparison between a conventional LAMP in a PCR machine and plasmonic thermocycling instrument was performed with Bst polymerase. LAMP functions at an optimal temperature range around 66^{o}C. This experiment demonstrates that PCR is not the only amplification technique possible by the plasmonic thermocycling method, but can be applied to a wide scope of methods.

LAMP Primers for F41 gene designed with Primer Explorer V4

ID	Tm	Sequence
F3	55.26	GCATATTACCAGTGGTTCTG
B3	55.85	GACTGAGGTCATCCCAATT
FIP		TTGAGAGTCCATTCCATTTATAGGC-TGGTACAGTATTTGAAGGGCTTG
BIP		AAGCTGAAATGGCTGGTTATGT-GTGGAAACCACTATAAGAGGTTGAA
F2	60.43	TGGTACAGTATTTGAAGGGCTTG
F1c	60.52	TTGAGAGTCCATTCCCATTTATAGGC
B2	60.54	GTGGAAACCACTATAAGAGGTTGAA
B1c	60.42	AAGCTGAAATGGCTGGTTATGT
LF	55.50	TCAGTAAATCGACCTGGATT
LB	55.72	TGCCAGGGAAAAGTGC

S7. References for Supplemental Material

1. M. L. Carnevale, P. J. R. Roche, M. Najih, M. Paliouras, L. K. Beitel and M. A. Trifiro, *Anal Methods-Uk*, 2015, **7**, 287-295.