Supplementary Information

Microfabrication-free nanoliter droplet array for nucleic acid detection combining with isothermal amplification

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1 Oligonucleotides and Reagents

Three different targets, the fragments originating from human OLR1, APOC2 and TP53 genes, were used to explore the performance of this method. All open circle probes (OCPs) and primers were evaluated using Primer 6.0 software to avoid secondary structure which may interfere probe function. Table 1 shows oligonucleotide sequences used in this study (Invitrogen Biotechnology Co., Ltd, Shanghai, China). All chemical reagents were analytical reagent grade.

Table S1 Sequences of targets, OCPs and primers used in the study.

Genes	Oligonucleotides	Sequences (5′->3′)
OLR1	Target1	TTTTGATTCTAGCTACCTGTATTATTT
	OCP1	PO4-TAGCTAGAATCAAAAGGGGTAACGGTGTGATATGAGCGTCGTATGGACGTGCAAGTTACCGAGCTGAAAATAATACAGG
	Forward primer1	CATACGACGCTCATATCACACCGT
	Reverse primer1	TACAGGTAGCTAGAATCAAAAGGGG
APOC2	Target2	CGGAAGTGGGTCTCAACCACTATAAAGCCTCTCTGTGC
	OCP2	PO4-TTTATAGTGGTTGAGACCCACTTCCGTGAACGACGAATCTGTACCATGCTAATGCGGCGTGATGTATTATGCGTATGAGCACAGAGAGGC
	Forward primer2	ATTAGCATGGTACAGATTCGTCGTTCA
	Reverse primer2	GCGGCGTGATGTATTATGCGTATGA
TP53	Target3	GCGTGTTTGTGCCTGTCCTGGGAGAGACTGGCGCACAGAGGAAGAG
	OCP3	PO4-GTCTCTCCCAGGACAGGCTTTCATTTACAGTTTACGGTTTAGCATTTCATTACTTTCATTTACTTTACGATTTCGGCTCTTCCTCTGTGCGCCA
	Forward primer3	AAATGAAAGCCTGTCCTGGG

Reverse primer3	CTTTACGATTTCGGCTCTTC

2 Preparation of hydrophobic surface

A standard glass slide was used as hydrophobic substrate on which nanoliter droplet array was generated. Specifically, after thoroughly cleaning with detergent solution and deionized distilled water (ddH_2O), glass slide was sequentially placed into acetone for 10 minutes, ethanol for 10 minutes, and isopropanol for 10 minutes, and each step was repeated twice. Then, the glass slide was rinsed thoroughly with ddH_2O. After dried with nitrogen, the slide was treated with oxygen plasma(Harrick Plasma, NY, USA) at medium position for 10 minutes. Subsequently, the glass slide was silanized by immerging into toluene containing octadecyltrichlorosilane (0.5 vol%) (Sigma-Aldrich, MO, USA) for 30 minutes. Then, the silanized slide was washed thoroughly using toluene for 10 minutes followed by isopropanol for 10 minutes. Finally, the slide was rinsed with ddH_2O to remove the residual chemical reagents thoroughly. The hydrophobicity of slide surface was estimated by observing the static contact angle of 1μl of ddH_2O dropped on the slide surface.

3 Formation of hydrophilic-hydrophobic pattern

Following hydrophobization, 0.2 mg/ml of BSA solution containing 1μM of primer pair was prepared and spotted onto the hydrophobic glass slide according to pre-designed patterns using a commercial microarray dispenser (Biodot, CA, USA) as 0.5 μl solution per spot. Then, the slide containing BSA solution spots was allowed to dry under room temperature, leaving BSA and primers on the hydrophobic glass slide surface. Finally, the dried BSA absorbed on silanized slide surface enable it produce hydrophilic spot array surrounded with hydrophobic coatings, and the primers was immobilized in the BSA matrix on the hydrophilic spots. The chip printed with primers was stored at 4 °C, ready for sample loading.

4 Templates preparation for HRCA reaction

Circle templates for HRCA reaction were prepared by ligation of OCPs and related targets. Reaction was set up in tubes with a 10μl reaction volume containing 1U Ampligase (Epicentre Technologies, Chicago, USA), 20 mM Tris-HCl (pH 8.3), 25 mM KCl, 10 mM $MgCl_2$, 0.5mM NAD, and 0.01% Triton X-100. Standard reactions including 1 nM OCPs and 1μl of desired concentration of targets were performed at 95 °C for 5 minutes, followed by 55 °C for 1 hour. After ligation, 5 U Exonuclease I (New England Biolabs, Ipswich, MA) was directly added into the tubes and incubated at 37 °C for 2 hours to degrade uncircled OCPs, followed by 80 °C for 20 minutes to heat inactivate.

5 Sample loading

Different combinations of previously prepared circle templates at desired concentration were added into the HRCA reaction mixture consisting of 20 mM Tris-HCl (pH 8.8), 10 mM$(NH_4)_2SO_4$, 10 mM KCl, 0.1% Tween-20, 0.5 mM dNTPs, 1× Evagreen dye (Biotium Inc., CA, USA), and 0.2U/μl Bst polymerase (New England Biolabs, MA, USA). Then the solution including multiple

circle templates was dispensed onto each individual BSA hydrophilic spot printed unique primer pairs with a conventional pipette manually as 0.5 μl of solution per spot. The sample-loaded chip was then immediately covered with mineral oil to prevent evaporation. The assembled chip was placed on a flat aluminum heating block (Poxiwaer Corp., Suzhou, China), and the HRCA reaction was run at 65 °C. During amplification, a fluorescence image was captured at every one minute with one-second exposure in 30 minutes by Las 4000 mini Imager (Fujifilm Inc., Tokyo, Japan).

6 Data processing

After imaging, the background-subtracted fluorescence intensity of each droplet was analyzed with Genepix 6.0 software and plotted to amplification curve using Origin 8.0 software. For data analysis, a modified method was used to calculate the time of threshold, named as Tt value, which was similar to Ct value in real-time PCR. For each droplet, threshold was estimated using 10-fold of the standard deviation (SD) value of the first five measure points. Tt value was defined as the time point at which the increment of fluorescence intensity became greater than the threshold.

7 HRCA reaction in conventional PCR tube

The components for HRCA reaction in conventional PCR tube were the same as the nanoliter droplet array assay, containing a final concentration of 1μM of specific primer pairs, except that the final reaction volume was 20μl. Then the reactions were incubated at 65°C and were real-time monitored for 30 cycles with a one-minute period on LightCycler 480 (Roche Corp., Basel, Switzerland). All reactions were run in triplicate.

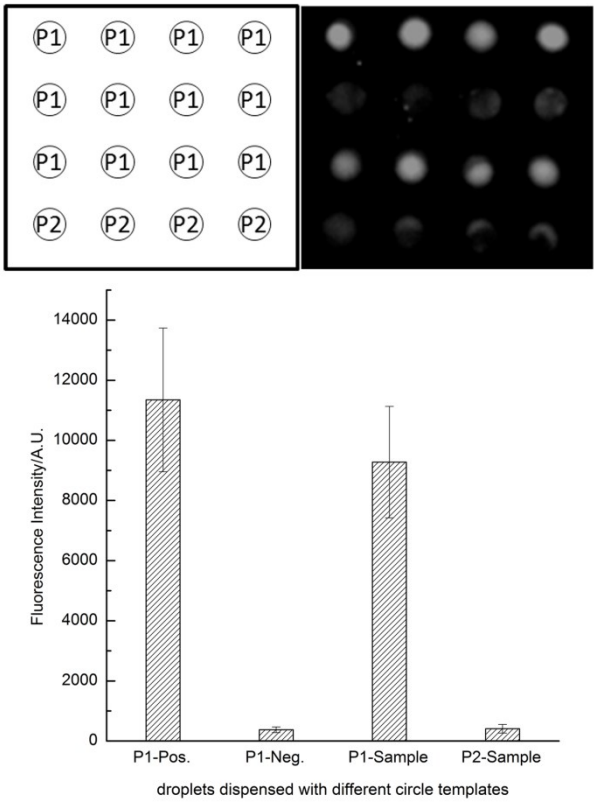

Figure S1. Detection of OLR1 gene from human genomic DNA extracted from A549 cell line. P1 represented the primer pairs matching with OCP related to OLR1 gene, and P2 represented the mismatched ones. The droplets dispensed with the appropriate templates (row 3) had a significant increase in fluorescent signal. The fluorescence signal of the droplets preloaded with mismatched primers (row 4) was very low after HRCA. The synthetic OLR1 cDNA and no target were used for positive controls (row 1) and negative controls (row 2) for detecting OLR1 gene target, respectively.