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Short Communication

Minimizing inhibition of PCR-STR typing using digital agarose droplet microfluidics

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ABSTRACT

The presence of PCR inhibitors in forensic and other biological samples reduces the amplification efficiency, sometimes resulting in complete PCR failure. Here we demonstrate a high-performance digital agarose droplet microfluidics technique for single-cell and single-molecule forensic short tandem repeat (STR) typing of samples contaminated with high concentrations of PCR inhibitors. In our multifaceted strategy, the mitigation of inhibitory effects is achieved by the efficient removal of inhibitors from the porous agarose microgel droplets carrying the DNA template through washing and by the significant dilution of targets and remaining inhibitors to the stochastic limit within the ultralow nL volume droplet reactors. Compared to conventional tube-based bulk PCR, our technique shows enhanced (20×, 10×, and 16×) tolerance of urea, tannic acid, and humic acid, respectively, in STR typing of GM09948 human lymphoid cells. STR profiling of single cells is not affected by small soluble molecules like urea and tannic acid because of their effective elimination from the agarose droplets; however, higher molecular weight humic acid still partially inhibits single-cell PCR when the concentration is higher than 200 ng/μL. Nevertheless, the full STR profile of 9948 male genomic DNA contaminated with 500 ng/μL humic acid was generated by pooling and amplifying beads carrying single-molecule 9948 DNA PCR products in a single secondary reaction. This superior performance suggests that our digital agarose droplet microfluidics technology is a promising approach for analyzing low-abundance DNA targets in the presence of inhibitors.

1. Introduction

PCR is an essential DNA analysis tool for forensic identification, clinical diagnosis, infectious disease detection, environmental monitoring, and archeological testing. [1,2] However, the presence of naturally occurring impurities in low-quality biological samples can lead to different degrees of PCR inhibition (i.e. underestimation of DNA quantification, multiplex peak imbalance, and allele dropout) and even false-negative results, posing a big challenge especially in the DNA analysis of limited samples. [3] A wide range of PCR inhibitors (e.g. humic acid, urea, heme, lactoferrin, collagen, melanin, indigo dye, tannic acid, and calcium ions) have been identified in complex specimens collected from various sources including soil, urine, blood, bone, hair, clothing, plant materials, food, etc. [3–5] Other inhibitors (e.g. detergent, guanidinium, salts, ethanol, isopropanol, phenol and powder from gloves) may also be introduced into the assays during the sample handling and DNA preparation processes. [6] These substances inhibit amplification reactions primarily through interference with DNA polymerases and their cofactors or direct interaction with template DNA and primers. [7,8]

A variety of strategies have been developed to overcome PCR inhibition. These techniques can be generally divided into two categories: (1) novel DNA extraction procedures to purify the target DNA prior to PCR amplification; and (2) modification of the DNA amplification process to minimize the effect of inhibitors that are not removed. The pre-PCR removal methods produce PCR-compatible DNA samples using silica-based extraction [9–13], magnetic separation [14–16], column chromatography [17–19], and other new protocols [20,21]. Mitigation of inhibition during PCR can also be achieved by extensive dilution of DNA extracts [22,23], utilization of genetically engineered DNA polymerases with better resistance to inhibitors (e.g. Ampli*Taq* Gold DNA polymerase used in commercial forensic kits) [24] together with appropriate buffer formulations [25–28], and addition of amplification facilitators such as bovine serum albumin (BSA) [22,23,29,30], T4 gene 32 protein [29,30],

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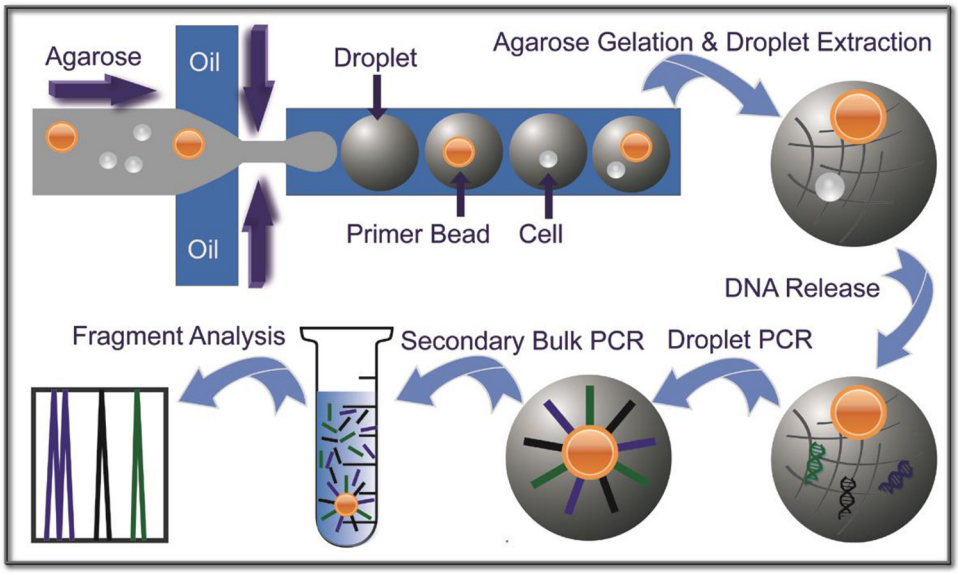

Fig. 1. Workflow for single-cell forensic STR typing using microfluidic agarose droplets. Single cells are encapsulated within nanoliter agarose droplets together with primer-functionalized beads using a microfluidic droplet generator. Following agarose gelation, the gel droplets are extracted from the oil phase, and single-cell genomic DNA is released into the gel droplet matrix through chemical lysis of the cells. The gel droplets are then equilibrated in PCR mixture and redispersed in oil by mechanical agitation; massively parallel droplet PCR is then implemented to amplify the STR targets from individual cells and to transfer the STR information onto the coencapsulated beads. Subsequently, beads are recovered by melting the agarose and statistically diluted to perform the secondary PCR amplification of individual beads. The STR products produced in free solution by the secondary PCR reaction are detected by conventional capillary electrophoresis fragment size analysis.

betaine [30], etc. Sample dilution is the simplest strategy to reduce the concentration of PCR inhibitors below the threshold of inhibition effects. However, dilution may not be practical with low concentration samples, because the DNA template may also be diluted below the detection limit.

Our previous work reported a highly sensitive and selective technique for single-cell and single-molecule short tandem repeat (STR) typing using nanoliter microfluidic agarose droplets (Fig. 1) [31]. In this method, agarose droplets are generated using microfluidic chips so that they have uniform size and droplet content, and thus act as efficient low-volume nL reactors for cell compartmentalization, cell lysis and single-genome extraction, and multiplex PCR. [31–35] Once encapsulated in agarose gel droplets, the cells are chemically lysed, releasing their genomic DNA into the gel droplet matrix. In the first round of droplet PCR, the STR information is efficiently transferred from a single cell to a coencapsulated primer-functionalized bead. Then the product-decorated beads are isolated from the emulsion and statistically diluted into conventional tubes for a second round of PCR amplification to produce replicas of STR products in free solution for conventional multiplex detection. Using this approach we successfully typed pure and mixed cell populations with single-cell resolution, high single-genome integrity and full allele recovery rates [31].

In the present study, we evaluate the ability of our agarose droplet-based single-cell/molecule STR typing technique to overcome the effects of PCR inhibition. The rationale for our approach is first that small molecule inhibitors can be washed out of the DNA-target containing gel droplets. Second, large inhibitors that might remain can be diluted along with the target DNA to the stochastic single copy limit (statistically), producing gel droplets

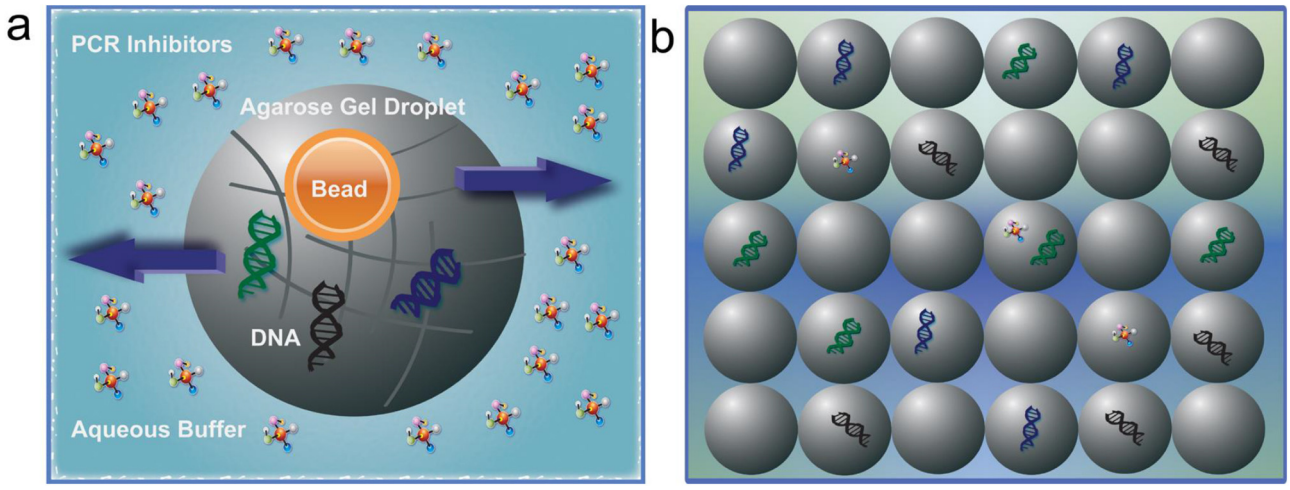

Fig. 2. Schematic illustration of how agarose droplets are used to remove or mitigate the effect of inhibitors in forensic DNA sample analysis. (a) PCR inhibitory molecules are efficiently removed from gel droplets during extensive washing steps via diffusion prior to performing droplet PCR, while high molecular weight genomic DNA remains entrapped within the agarose matrix. (b) Higher molecular weight PCR inhibitory molecules are statistically diluted among the ultralow volume (1.5 nL) droplets. At this stochastic limit, droplets that happen to contain template but no DNA inhibitor provide successful forensic PCR.