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Multiple pathogen biomarker detection using an encoded bead array in droplet PCR

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ABSTRACT

We present a droplet PCR workflow for detection of multiple pathogen DNA biomarkers using fluorescent color-coded Luminex® beads. This strategy enables encoding of multiple singleplex droplet PCRs using a commercially available bead set of several hundred distinguishable fluorescence codes. This workflow provides scalability beyond the limited number offered by fluorescent detection probes such as TaqMan probes, commonly used in current multiplex droplet PCRs. The workflow was validated for three different Luminex bead sets coupled to target specific capture oligos to detect hybridization of three microorganisms infecting poultry: avian influenza, infectious laryngotracheitis virus and *Campylobacter jejuni*. In this assay, the target DNA was amplified with fluorescently labeled primers by PCR in parallel in monodisperse picoliter droplets, to avoid amplification bias. The color codes of the Luminex detection beads allowed concurrent and accurate classification of the different bead sets used in this assay. The hybridization assay detected target DNA of all three microorganisms with high specificity, from samples with average target concentration of a single DNA template molecule per droplet. This workflow demonstrates the possibility of increasing the droplet PCR assay detection panel to detect large numbers of targets in parallel, utilizing the scalability offered by the color-coded Luminex detection beads.

1. Introduction

Pathogen detection techniques are crucial to assess the prevalence and control the spread of diseases. The need for automated methods for pathogen detection is not limited to human diseases. Pathogen monitoring in food production is essential to ensure food safety. Biomarkers such as cell surface proteins or unique sequences of DNA or RNA, are used for identification of specific pathogens. Nucleic acid biomarker sequences are commonly amplified by PCR and detected using sequence specific fluorescent probes, such as TaqMan probes. To increase assay sensitivity to single molecule detection, droplet-based PCR have been introduced. Droplet microfluidics employs surfactant stabilized monodisperse aqueous droplets in immiscible oil as reaction vessels. The picoliter-sized droplets are generated, processed and analyzed in microfluidic devices (Theberge et al., 2010; Joensson and Andersson Svahn, 2012). The high sensitivity of droplet PCR is achieved by partitioning the total DNA in a large number of droplets prior to amplification. The encapsulation of template DNA at limiting dilutions alleviates the amplification bias in droplet PCR, which limits the conventional PCR techniques (Williams, 2006). In addition, partitioning enables absolute quantification of target DNA without the need for external references. The number of TaqMan-positive and -negative droplets are counted based on their fluorescence signal at the end of the PCR. The droplet count is then used to determine the initial concentration of target DNA using Poisson distribution statistics. Droplet PCR has been used for detection of rare mutations (Pekin et al., 2011; Taly et al., 2013; Shuga et al., 2013), target genome enrichment before Next Generation Sequencing (Tewhey et al., 2009; Eastburn et al., 2015), screening of rare microbes (Lim et al., 2015) and genes (Fallah-Araghi et al., 2012), and a number of other applications.

A TaqMan PCR can be multiplexed to detect multiple biomarkers in the same assay by including several target specific TaqMan probes, each coupled to distinct fluorophores. Droplet PCR assays for detection of multiple targets in a sample has been demonstrated by varying the concentrations target-specific TaqMan probes (Taly et al., 2013; Zhong et al., 2011). However, the number of targets that can be analyzed in a single assay is practically limited to ca 5 targets, due to the spectral overlap between the probe fluorophores.

Moreover, multiplex PCR assays are difficult to optimize due to the multiple PCR reactions performed in a single vessel. Specifically, the

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development of a multiplex PCR must aim to limit PCR artifact generation and avoid bias in the amplification of all targets. The inherent complexity in a multiplex PCR makes modifying the detection panel increasingly more difficult with an increasing number of probes and requiring more extensive optimization, compared to singleplex assays.

Another strategy to multiplex reactions is to couple the amplified targets to an encoded bead-based array. Bead-based arrays have previously been used for droplet PCR readout with primer-conjugated agarose beads used along with fluorophore-labeled primers to amplify target DNA and capture the resulting labeled PCR amplicons on their surface (Kumaresan et al., 2008; Zeng et al., 2010). The beads were then analyzed in flow cytometer to detect the captured amplicons. However, the spectral overlap of fluorophores limits the number of detectable targets in the same manner as the TaqMan probe approach. Luminex suspension array technology offers 500 distinct color-coded beads sets to detect as many targets and is widely used for detection of multiple targets from a single sample (Schwenk et al., 2006; Navidad et al., 2013). Each Luminex bead contains a specific concentration of red and infrared dyes which makes each bead sets distinguishable from one another.

In this paper, we report a droplet PCR workflow that uses color-coded Luminex beads to detect pathogen DNA biomarkers (Fig. 1). Luminex bead based readout from droplet PCR allows concurrent detection of multiple biomarker targets with an exchangeable and extendable target panel. Nucleic acid biomarkers associated with some of the common microorganisms infecting poultry: avian influenza virus (AIV), infectious laryngotracheitis virus (ILTV) and *Campylobacter jejuni* (not pathogenic to poultry, but pathogenic to humans if zoonotically transmitted) were detected using this approach. These targets were selected as model target based on their clinical relevance. AIV outbreaks occur in many countries around the world (Sonnberg et al., 2013) and infections of *Campylobacter jejuni* have increased over the past years (Kaakoush et al., 2015). The presented hybridization assay showed high specificity and enabled detection of positive beads that has captured PCR-amplicons of the target DNA, from a background of negative beads. Accurate and rapid detection of pathogens present in poultry will help to control the spread of disease among birds. It will also help to prevent zoonotic pathogens from spreading to humans who come in contact with the affected poultry flocks. Partitioning the sample into picoliter droplets allows a multi target PCR assay to be divided into several singleplex reactions. With this readout strategy, multiple targets can be detected without the limitations of PCR amplification bias associated with multiplex PCR. It also reduces the optimization needed for introducing new targets, which leads to a more flexible and extendable detection panel.

2. Experimental

2.1. Microfluidic chip manufacturing

The microfluidic chips used in the experiments were fabricated by standard soft lithographic technique (McDonald et al., 2000). A 9:1 (w/w) mixture of polydimethylsiloxane (PDMS) and curing agent (Dow Corning Corp.) was poured on the silicon wafer containing SU-8 patterned channel structures. The mixture was degassed to remove air bubbles and cured overnight at 65 °C. The PDMS slab was cut and gently peeled off from the master. The inlet/outlet ports of the channels

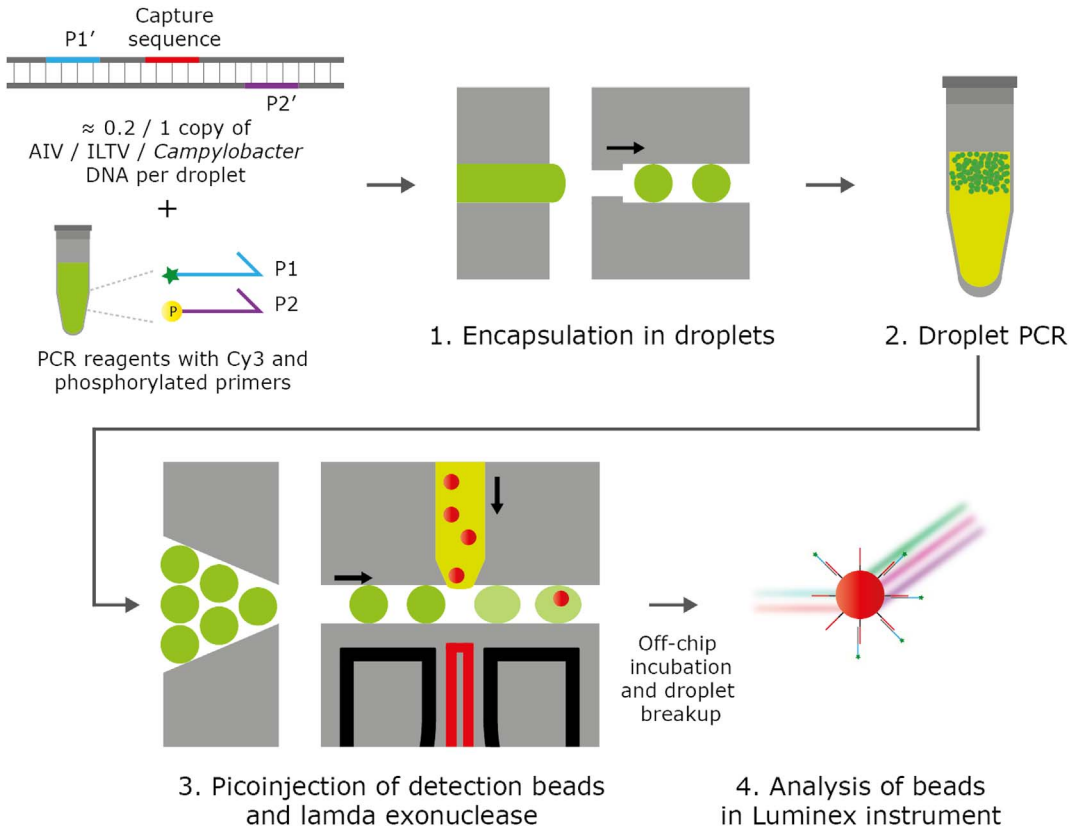

Fig. 1. Schematic workflow of color-coded bead based pathogen biomarker detection by droplet PCR. The target DNA was mixed with PCR reagents containing a set of Cy3-labeled and phosphorylated target specific primers. The reaction mixture (green color) was encapsulated in 9 pL droplets and collected in a PCR tube for thermocycling off-chip. The droplets (green circles) containing PCR product were re-injected into a picoinjection chip, where the droplets were injected with lambda exonuclease (yellow color) enzyme and detection beads (red circles). The emulsion was collected in a PCR tube and incubated to initiate the digestion of phosphorylated DNA strand by the enzyme. After hybridization of Cy3 labeled PCR product to the respective detection beads, the droplets were broken to recover the detection beads that were then analyzed in a Luminex instrument. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)