

HIGHLY SPECIFIC ZEPT-MOLE LEVEL DNA DETECTION BY COMBINATION OF THERMAL LENS MICROSCOPE AND ROLLING CIRCLE AMPLIFICATION

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ABSTRACT

We for the first time report the development of a DNA detection method which can quantify zept-mole level DNA by combination of Thermal Lens Microscope (TLM) and Rolling Circle Amplification (RCA). In this method, the target DNA is extended by RCA, and then the RCA products are detected by TLM, making use of high selectivity of RCA and high sensitivity of TLM. This method is more highly specific than PCR and the detection limit is 22 zeptomol. This study is contributable to cancer diagnosis and detection of viruses.

KEYWORDS: DNA, diagnostic, RCA, clinical use

INTRODUCTION

Recently, the quantification of DNA on zeptomole order has been widely required in the field of cancer diagnosis, study of viruses, etc. To meet this requirement, Rolling Circle Amplification (RCA) has been widely studied [1]. In RCA, the specific DNA strand is amplified and become a long strand. By being hybridized with fluorescent complementary DNA, the original DNA is detected as a fluorescent dot (Figure 1 top). This method has higher selectivity than PCR and distinguishes the single nucleotide difference. However, quantitative detection on zeptomole order has been difficult because the number of detected dots has no repeatability and relies on the threshold between noises and signals. On the other hand, we have previously developed a method of quantitative detection of non-fluorescent molecules by utilizing light absorption and thermal relaxation of the target molecule, which is called thermal lens microscopy (TLM) [2]. TLM is effective in concentration determination of liquid sample. Following this, the idea of this study is to quantify RCA products by TLM in liquid phase. However, the quantitative detection of RCA is difficult because the RCA products do not adsorb the visible light. Therefore, the challenge is to convert the RCA products into another chemical which has visible light absorption so that the concentration can be determined by TLM.

The objective of this study is to realize the chemical conversion process and to obtain the quantitativity of the zeptomole-order DNA with high selectivity.

THEORY

Rolling Circle Amplification is an isothermal method of extending the DNA. RCA starts when the target DNA is caught by the padlock probe hybridized with the primer. Then, the edges of padlock probes are ligated by ligase and the padlock probe becomes a circle template. Subsequently, the primer is extended by polymerase and becomes the long single strand DNA with repeated gene arrangement (RCA product, Figure 1 left). As the conventional method, the complimentary DNA with fluorescent dye is hybridized to the RCA products and they are detected by fluorescent microscope (Figure 1 top). Because one dot is originated from one target DNA, the amount of target DNA is quantified by counting the number of fluorescent dots on solid surface.

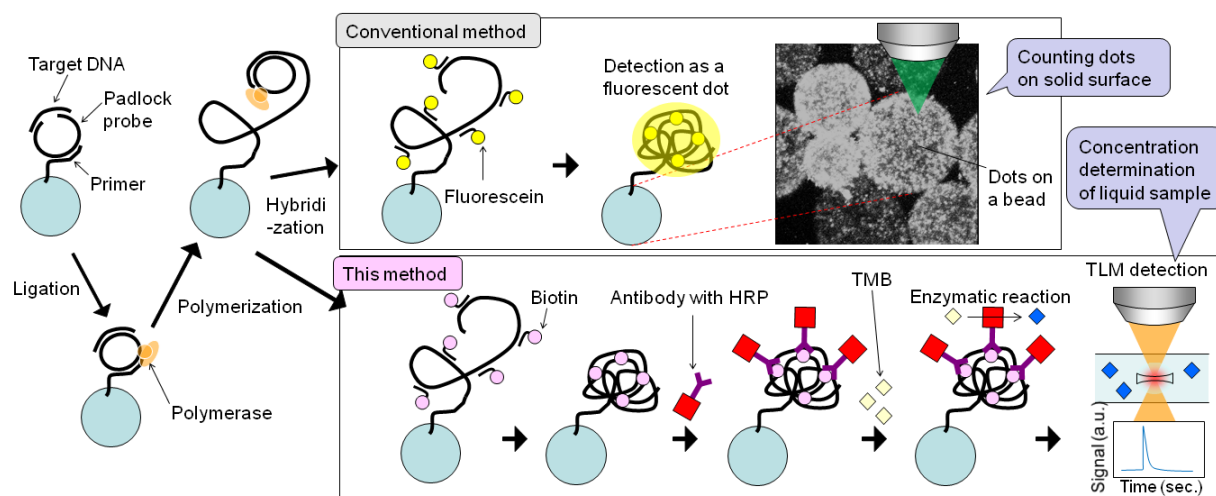


Figure 1: Scheme of RCA, enzyme modification and TLM detection compared to conventional method

On the other hand, the idea of this study is to detect the RCA products quantitatively in liquid phase by TLM. However, technically TLM cannot detect the sample which has no absorption in visible range. In order to detect the RCA products by TLM, we propose the chemical conversion process below (Figure 1 bottom). First, the long strand DNA is hybridized with biotin-modified complimentary DNA. Subsequently, anti-biotin-antibody with Horseradish peroxidase (HRP) combines to biotin and the whole long strand is modified with HRP. We measured the amount of the target DNA by measuring the enzymatic activity of HRP by TLM.

EXPERIMENT

To confirm the principle, padlock probe is used as the target DNA. The scheme of procedure is shown in figure 2. Prior to observe the concentration dependence, the signal amplification by RCA is confirmed by varying the polymerization time. In the PCR tube, padlock probes at 1nM were mixed with ligation solution and primer-immobilized agarose beads (34 μ m as a diameter), and then set at 30°C for 30 minutes to allow hybridization and ligation of padlock probes. After 30 minutes, the polymerization solution is added to the PCR tube and set at 30°C for varying polymerization time (0, 5, 30, 60, 90, 180 minutes). After polymerization process, the beads with extended DNA were mixed with hybridization solution including 100nM biotin-modified complimentary DNA and incubated for 30 min (50°C, 40°C, and 30°C, each for 10min). After the hybridization of biotinylated complimentary DNA, the beads were injected into the microchip-based immunoassay system called μ ELISA system [3] (Figure 3(a)). μ ELISA system has a built-in syringe pump in it and can inject or pump a reagent solution automatically. In addition, it has thermal lens microscope in the downstream (Figure 3(b)). In this system, about 2000 beads were injected and accumulated in the channel. Then, Anti-biotin IgG with horseradish peroxidase (HRP) was injected into the channel before the solution of Tetramethylbenzidine (TMB) and H₂O₂. In the channel, H₂O₂ oxidized TMB by catalytic action of HRP and the thermal lens signal of oxidized TMB was detected as a peak. The excitation wavelength was 658nm. After confirmation of signal amplification, we conducted the experiment with several concentrations of the target DNA (0nM, 0.1nM, 0.25nM, 0.5nM, 1nM).

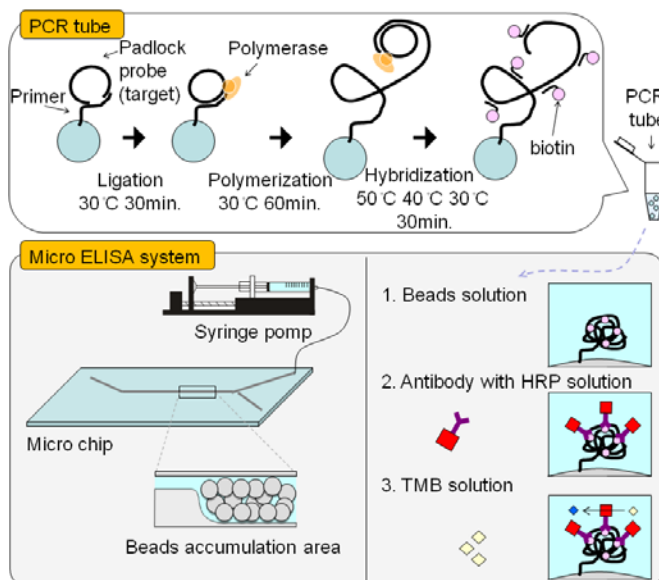


Figure 2: Schematic diagram of protocol

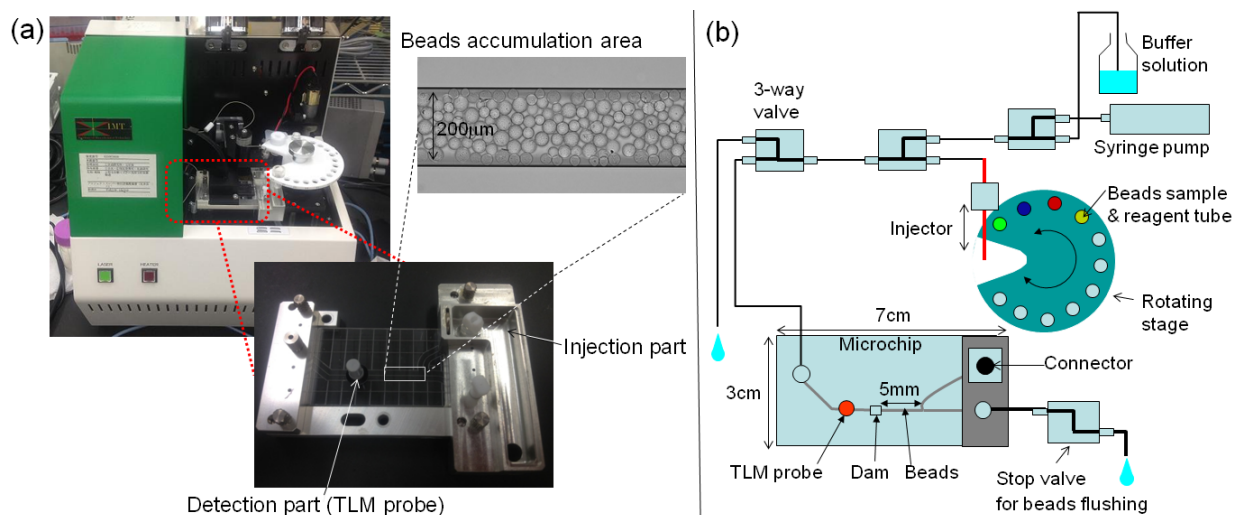


Figure 3: (a) Photo of detection system and microchip (b) Schematic diagram of pumping system of Micro ELISA system

RESULTS AND DISCUSSION

The results are shown in Figure 4 and 5. Figure 4 shows the result of the experiment of varying polymerizing time. The concentration of padlock probe is 1nM. Because of the positive correlation between polymerization time and thermal lens signal, it is confirmed that the signal gained by this system is from the RCA products. In addition, signal amplification by RCA is confirmed when the polymerization time is more than 30 minutes. It is still not clear why it needs 30 minutes the thermal lens signal is amplified by RCA. Herein, we optimized the polymerization time at 60 minutes.

Figure 5 shows the linear correlation between the concentration of padlock probe and thermal lens signal. The detection limit was 22 zeptomole. Accordingly, the quantitativity on the order of 10 zeptomole is confirmed for the first time. In order to improve the detection limit, further decrease of background is necessary. The main cause of background would be non-specific adsorption of HRP. Therefore decreasing the background can be achieved by optimizing the beads material or the blocking method.

This method will contribute to genetics, oncology, and virology.

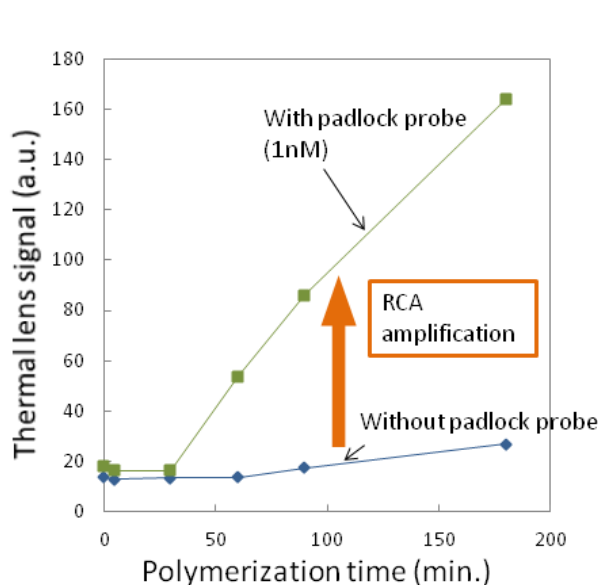


Figure 4: Correlation between the polymerization time and thermal lens signal. Blank (blue) and 1nM (green) of padlock probe concentration is shown.

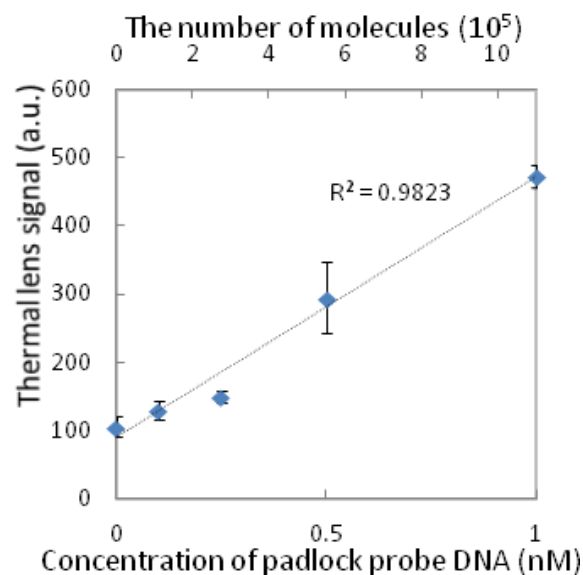


Figure 5: Correlation between thermal lens signal and the concentration of padlock probe. The corresponding numbers of molecules are shown in the top.

CONCLUSION

In this report, we developed a novel quantifying method of DNA utilizing Rolling Circle Amplification (RCA) and thermal lens microscope (TLM). Although RCA has higher selectivity than PCR, the quantitativity of zeptomole order is not confirmed. In order to realize the quantification on zeptomole order, we think of detecting the RCA products by TLM. However, detecting RCA products by TLM directly is difficult because the RCA products do not have light absorption in visible range. To cope with this, we introduced the chemical conversion process and succeeded in detecting the RCA products by TLM. The detection limit was 22 zeptomole. We believe that this method will contribute to cancer diagnosis and detection of viruses.

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