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Short communication

Preliminary evaluation for a novel voltammetric analysis of targeted nucleic acid by combining electrochemical DNA chip and digital loop-mediated isothermal amplification

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ABSTRACT

We propose a novel voltammetric analysis method for the targeted nucleic acid quantification using electrochemical DNA chip and digital loop-mediated isothermal amplification (LAMP), which is based on the most probable number (MPN) method. We designed the position of electrodes (30 regions) on the DNA chip surface at 2 mm intervals in the flow channel. Since the LAMP reaction is carried out at constant temperature, it is supposed that the motions of both the targeted nucleic acid and amplicon in the LAMP solution are diffusion-limited. So an independent volume is formed around the electrode (like a test tube). MPN for a single dilution with any positive electrodes was given by MPN (μL^{-1}) = $1 / m_e \times 2.303 \times \log$ ((number of total electrode) / (number of negative electrode)). The m_e denotes the amount of sample in each volume covered by an electrode on the DNA chip. When different concentrations of targeted nucleic acid were amplified, all electrodes on the DNA chip were positive above 2×10^1 copies/μL. On the other hand, positive ratios of detection decreased to 25% and 88% at 2×10^{-1} and 2×10^0 copies/μL, respectively. From these results, the volume covered by an electrode in the flow channel was calculated to be 1.2 ± 0.2 μL. The MPN method is applicable to nucleic acid analysis using electrochemical DNA chip and several copies of targeted nucleic acid are quantified precisely without calibration curve.

1. Introduction

Quantitative analysis of targeted nucleic acid such as pathogen detection and RNA expression analysis is widespread in molecular testing. Real-time polymerase chain reaction (PCR) is the most popular method for quantifying targeted DNA or RNA molecules [1,2]. However, it requires specialized fluorochrome and an expensive optical monitoring system. Furthermore, the preparation of the calibration curve for each targeted molecule is necessary for quantification and the precision of quantitative analysis for a tiny amount of nucleic acid is low. Digital PCR has been developed recently to overcome these problems. Highly precise quantitative analysis is possible by direct counting of the nucleic acid molecules without preparation of the calibration curve [3,4]. However, complex pretreatment is needed to divide nucleic acid molecules from one another using small droplets or fine wells and expensive detection equipment is necessary for the counting. Therefore, realization of a simple, low-cost nucleic acid quantification method is still required.

Electrochemical methods for nucleic acid detection have been developed to realize a portable device and low-cost testing [5–7]. Real-time electrochemical nucleic acid detection was achieved recently using an intercalative redox compound [8–10]. Although use of a disposable electrode and a compact electrode detector makes possible highly sensitive quantitative analysis of targeted nucleic acid [11], the calibration curve is still needed for quantification.

We have been developing a device for electrochemical monitoring of the loop-mediated isothermal amplification (LAMP) reaction [12–14] using a DNA chip consisting of plural electrodes and a flow channel [15], and found that there are both positive and negative electrodes when lower concentration of targeted nucleic acid was reacted (< 100 copies). We speculated that nucleic acid molecules hardly move in a narrow flow channel in the case of LAMP reaction at constant temperature and the reaction volume covered by an electrode is controllable by temperature, viscosity and reaction time. So it is assumed that the regulated reaction volume covered by an electrode in a flow channel acts as an independent test tube.

The most probable number (MPN) method has been widely used for microbial density estimation in water or foods since the early twentieth century. The MPN method is useful for low concentrations of bacteria (< 100). It is not necessary to prepare the calibration curve for

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quantification [16,17]. We supposed that the enzymatic amplification of targeted nucleic acid in a microtube resembles the growth of bacteria in a test tube. So we propose a new quantification method for the targeted nucleic acid using electrochemical DNA chip and the LAMP reaction, which is based on the MPN method. In this paper, we report the preliminary evaluation of the novel method and its possibilities for highly precise and highly sensitive quantification of targeted nucleic acid.

2. Materials and methods

2.1. Materials

A plasmid DNA having parvovirus VP2 gene was used as a model for verification of the novel nucleic acid quantification method. LAMP primers used are described in Table S1. The electrochemical DNA chip, consisting of 30 regions of gold electrodes (Φ = 200 μm) patterned on a Pyrex glass substrate, silicone packing for liquid flow channel (W1 mm × H1 mm × L50 mm), and a plastic cassette, was purchased from Toshiba Hokuto Electronics Corp. (Fig. S1) [18].

2.2. Methods

2.2.1. LAMP reaction condition

The LAMP reaction was carried out at 65 °C by mixing 1.6 μM each of FIP and BIP primer, 0.12 μM each of F3 and B3 primer, 0.4 μM of Lb primer, 1.4 mM dNTPs, 20 mM Tris-HCl (pH 8.0), 60 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween20, 0.8 M betaine and 0.8 units/μL of the GspSSD DNA polymerase unless otherwise noted. 50 μL of the LAMP solution containing 1 mM ruthenium hexamine (RuHex) was used for the electrochemical indicator of the LAMP reaction.

2.2.2. Electrochemical measurement

The electrochemical DNA chip was mounted on a customized holder and heated at 65 °C. The electrochemical signal of RuHex in the LAMP solution was measured by electrochemical analyzer ALS620e and multiplexer ALS684 (ALS Co., Ltd). The measurement conditions for linear sweep voltammetry (LSV) were: initial voltage = 0.1 V, final voltage = − 0.4 V, scan rate = 0.5 V/s. The cathodic reaction of RuHex was recorded for 60 min at every minute. The peak current was determined by ALS 600 series software (version 14.08J). According to the LAMP reaction of the positive sample, the cathodic peak current of RuHex increased [15]. So we estimated the relationship between the initial number of copies of the targeted nucleic acid and the time when the cathodic current began to increase (Fig. S2).

3. Results and discussion

3.1. Principle of novel nucleic acid quantification using MPN method

Fig. 1 shows a schematic diagram of the novel nucleic acid quantification method (top view: A and B, side view: C). The plural gold electrodes (30 regions, 2 mm intervals) exist in the 50 μL of flow channel. The LAMP reaction is carried out in the electrochemical DNA chip cassette after injection of the solution including the targeted nucleic acid. In the case of high concentration of targeted nucleic acid, all electrodes show positive signal because they exist around all electrodes on the DNA chip (Fig. 1A). On the other hand, some electrodes are only positive when low concentration of targeted nucleic acid is reacted (Fig. 1B). Since the LAMP reaction is carried out at constant temperature, it is supposed that the motions of both the targeted nucleic acid and amplicon in the LAMP solution are diffusion-limited. In aqueous solution, diffusion coefficient of the DNA fragment (6000 base pairs) was reported to be 0.81×10^{-8} cm^2/s [19], and so diffusion distance of DNA fragment is calculated to be several hundred meters during the LAMP reaction for 60 min. Here, we suppose that each electrode occupies an independent area on the DNA chip surface like a test tube under a constant LAMP reaction temperature (Fig. 1C) and the MPN method is applicable to quantification of targeted nucleic acid.

MPN for single dilution with any positive samples is given by Blodgett [20].

$$MPN = (1/m) \times 2.303 \times \log_{10}((\ t_j m_j)/(\ (t_j - g_j)m_j))$$

m denotes the amount of sample in each tube in the dilution with a positive tube,
$\Sigma t_j m_j$ denotes the amount of sample in all tubes in the selected dilutions, and $\Sigma(t_j - g_j)m_j$ denotes the amount of sample in all negative tubes in the selected dilutions.

MPN (copies/μL) using the DNA chip is simply expressed by

$$MPN = 1/m_e \times 2.303 \times \log((\text{No. of total electrodes})/(\text{No. of negative electrodes})) \quad (1)$$

m_e denotes the amount of sample in each volume covered by an electrode on the DNA chip.

The concentration of targeted nucleic acid is statistically determined by detection of the positive ratio using the electrochemical DNA chip after the LAMP reaction. The preparation of calibration curve is unnecessary for quantification.

To determine the MPN by Eq. (1), it is necessary to know the volume covered by an electrode on the DNA chip. To determine it, different concentrations of targeted nucleic acid (2×10^{-1}, 2×10^{0}, 2×10^{1}, 2×10^{2}, 2×10^{3}, 2×10^{4} copies/μL) were amplified at 65 °C and the cathodic current of RuHex was measured simultaneously up to 60 min using LSV for quantitative analysis. The eight electrodes on the DNA chip were used for data analysis. Fig. 2 showed the relationship between the initial number of copies of the targeted nucleic acid and the time when the cathodic current began to increase. All electrodes on the DNA chip were positive above 2×10^{1} copies/μL. On the other hand, positive ratios of detection decreased to 25% and 87.5% at 2×10^{-1} and 2×10^{0} copies/μL, respectively. From Eq. (1), m_e is given by

$$m_e = 2.303 \times \log((\text{No.of total electrodes})/(\text{No.of negative electrodes}))/\text{MPN}$$

m_e calculated by the data of 2×10^{-1} copies/μL and 2×10^{0} copies/μL were 1.0 μL and 1.4 μL, respectively. The volume covered by an electrode on the DNA chip is determined as 1.2 ± 0.2 μL.

Then we prepare the conversion table from the results of electrochemical DNA chip to MPN and 95% confidential intervals using different numbers of electrodes (8 and 80) on the DNA chip without dilution (Table 1). When the sample is diluted by X fold, the actual MPN is calculated by X times. It is shown that the precision of quantification value is increased by increasing the number of electrodes.

3.2. Design of electrochemical DNA chip suitable for MPN method

The size of flow channel used in this experiment is W1 mm × H1 mm × L50 mm. So it was supposed that an electrode covers an area of 0.6 mm in an anteroposterior direction from its center because the volume covered by an electrode on the DNA chip is determined as 1.2 μL. To realize the high-precision testing with a smaller DNA chip, it is preferable to increase the density of electrodes. However, narrow space between electrodes has a risk to cause a decrease in precision of quantification due to the confusion as to the volume covered independently by each electrode. It was supposed that the interval of electrodes > 1.2 mm is preferred for highly precise quantification.

We calculated the motion of nucleic acid molecules in the LAMP reaction at 65 °C for 60 min. Fig. S3 shows the gel electrophoresis image of the LAMP reaction. The size of amplicons ranged from 200 to