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Precise Dispensing Technology Using Electroformed Tubes for Micro-Volume Blood Diagnosis

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ABSTRACT Precise dispensing of droplets is a crucial step for acquiring reliable blood diagnostic results. When the source sample volume is limited, the need for precise dispensing of submicroliter and nanoliter quantities is especially important. In this paper, we developed a positive-displacement high-precision dispensing technique using a nickel electroformed tube with an inner diameter accuracy of 5 μm or less. When dispensing variation of 100 nL was evaluated by using a photometric method, the most stable coefficients of variation (CV) were observed for a tube thickness of 50 μm with hydrophobic treatment, where the average CV value was 1.3%, *i.e.*, 1.3 nL. Furthermore, the glucose concentration of 100- and 200-nL animal-based control serum was colorimetrically measured using enzymatic reactions without drying and mixing reagents. The CV value was approximately 6.36% at 100 nL and 3.23% at 200 nL, suggesting that several biochemical panels can be precisely measured even from less than one drop of blood. This positive-displacement dispenser ensures zero contamination and almost-zero dead volume, thus it would be useful for multi-panel clinical blood testing.

INDEX TERMS Blood testing, POCT, dispensing, biochemical analysis, glucose.

I. INTRODUCTION

Emerging platforms for detecting rare biomarkers and tumor cells in the bloodstream have increasingly improved diagnostic sensitivity [1]–[5]. Therefore, early signs of metabolic changes can be detected even with a tiny biosample. When a micro-volume sample with less than a few microliters is used for a clinical diagnosis, precisely manipulating and dispensing submicroliter and nanoliter droplets is one of the most crucial steps for acquiring reliable multi-panel results. This is particularly true when the collected volume is extremely limited such as blood samples of newborns and elderly persons. In general, such liquid handling technologies have been developed for the development of new drugs for pharmaceutical companies, analysis of DNA sequences to monitor gene activity, immunoassays, lab-on-a-chip applications, and cell sorting [6]–[8]. However, there are some problems in achieving precise dispensing on a nanoliter scale with zero cross-contamination and minimum dead volume. For example, dispensing volumes around 100 nL can be challenging because the dispensing process is dominated by interfacial adhesion and factors, such as surface tension, capillary forces, and local microstructures, that could affect the transferred volume [9], [10].

Liquid handling technologies can be broadly classified as noncontact and contact dispensing methods. Noncontact methods such as piezo-actuated and acoustic droplet ejection allow high-throughput and non-contamination dispensing, but have difficulty in the precise volume control of ejected solutions when the liquid has various viscosities. Contact dispensing is useful for nanoliter and microliter volumes because it is a simple and reliable method of touching the wall of a container for dispensing liquid. One of the most stable techniques for dispensing is to use a positive-displacement-type pipette with a plastic tube and steel core plunger, which works as a piston without an air gap [11]. This method of using a disposable tip allows for a relatively precise dispensing with zero cross-contamination and almost-zero dead volume even if the solution has viscosity. However, with the currently used resin pipes, the machining accuracy is limited to about several tens of μm, and there is a possibility of plastic deformation

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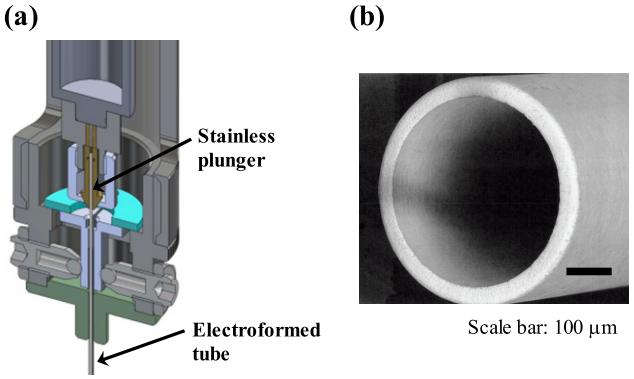

FIGURE 1. (a) Schematic of dispenser and (b) scanning electron microscope image of electroformed tube.

by thermal expansion and mechanical attachment; therefore, the best coefficients of variation (CV) value for submicroliter dispensing would be about 5%.

In the present work, we developed a positive-displacement high-precision dispensing system using a nickel electroformed tube for a point-of-care multi-panel diagnosis with a micro-volume blood sample. As a feasibility study, we investigated the design of electroformed tubes for precise dispensing and measurement stability of glucose concentration in animal-based control serum.

II. EXPERIMENTAL PROCEDURES

A. DISPENSING TECHNIQUE USING PRECISE ELECTROFORMED TUBES

For our positive-displacement dispensing method, a nickel electroformed tube and a stainless plunger were used. An electroformed tube was manufactured by casting an electroformed layer on a metallic wire, which was subsequently removed. With this method, the inner diameter of the tube can be strictly controlled by a selected outer diameter of the wire. Electroformed tubes with three kinds of inner diameter precision were prepared here; the standard deviation of the inner diameter was set to either less than 1, 10, or 30 μm. Also, the tip angle of the tubes was changed to 0, 10, and 20°, which refers to the angle formed by the bottom surface of a microplate well and the end surface of an electroformed tube. The dependencies of dispensing precision on inner diameter precision and tip angle were investigated for optimization.

The transmission image of the manufactured electroformed tube was captured with X-ray computed tomography (CT) (Zeiss, Xradia 520 Versa; resolution, 0.7 μm per pixel) and the accuracy of the inner diameter was measured by image analysis. The desired inner diameter was set to 500 μm and the tube length was 40 mm. The length of the plunger (outer diameter tolerance, <1 μm) was set to 50 mm, and the outer diameter was adapted in accordance with the inner diameter precision used.

Figure 1 shows a schematic of our developed dispenser and a typical scanning electron microscope image of the edge of an electroformed tube. To precisely drive the plunger

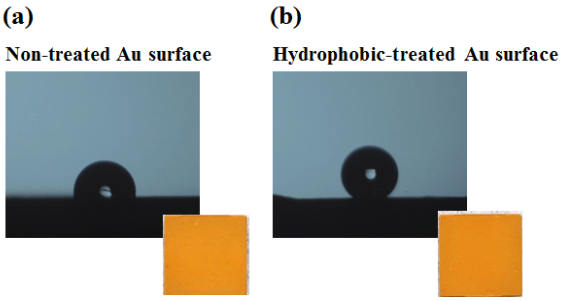

FIGURE 2. Difference of water contact angles on Au surfaces (a) without and (b) with hydrophobic treatment. The inserted photos are the outside appearances.

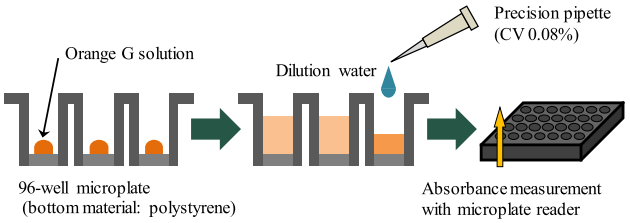

FIGURE 3. Procedure for evaluating variation in dispensed volume.

for dispensing, we developed a driving mechanism that can directly connect the stepping motor and the plunger. The driving mechanism of a stepping motor and a plunger is similar to that of drill chucking. A steeping motor has a metallic rod in the tip, and the plunger is fixed in the tip when a connector cap is screwed. Thus, the stepping motor and the plunger move strictly together so that the motor precision directly effect on the plunger precision. A stepping motor with a minimum resolution of 0.04 μm was used to acquire strict stepping accuracy.

To prevent time-dependent surface changes in the nickel and stainless steel due to oxidation, gold plating was applied to the outer surfaces of the nickel electroformed tube and the edge face of the plunger. Moreover, to reduce liquid retention on the edge of the electroformed tube, the outside surfaces of the tube were hydrophobically treated with self-assembled monolayers on a gold surface [12], [13]. When the hydrophobic treatment was applied to the surface of a gold substrate equivalent on the outer surface of the tube, the static contact angle of water showed a reduction in liquid retention changing from 86.7 to 141.7 degrees (Fig. 2).

B. ASSESSMENT METHOD FOR MEASURING DISPENSED SAMPLE VOLUMES

A conversion method for dye concentration was used for the evaluation of the dispensed volume [14]. As shown in Figure 3, 100 nL of Orange G solution (Wako Chemical) was dispensed into 16 wells of a 96-well microplate (Thermo Scientific). After dilution in 200 μL of water, the absorbance (corresponding to dispensed volume) was measured with a microplate reader (Infinite F200 PRO, TECAN). The dilution water was transferred with a high-precision automatic pipette (CV 0.08%), and the absorbance was measured at a

wavelength of 492 nm after sufficient mixing. The CV value was calculated from the absorbance of n = 16 measured in the above manner, and the value corresponds to N = 1. The accuracy of the dispensed volume can also be measured by this method, but since the volume can be easily changed by adjusting the stepping motor, the accuracy was evaluated only as a reference data.

C. MEASUREMENT OF GLUCOSE CONCENTRATION IN ANIMAL-BASED SERUM

To investigate the feasibility of using our developed precise dispensing system for clinical diagnosis with micro-volume blood, the glucose concentration in 200 nL of precisely dispensed animal-based control serum (Autonorm, Sekisui Medical) was measured, and the analytical CV values were calculated. Glucose is a key diagnostic parameter for many metabolic disorders [15], [16]. For instance, increased glucose levels have been associated with diabetes mellitus, hyperactivity of thyroid, pituitary, and adrenal glands. Conversely, decreased levels are found in insulin secreting tumors, myxedema, hypopituitarism, and hypoadrenalism. For the measurement, a glucose assay kit (EBGL-100, BioAssay Systems) was utilized in accordance with the manufacturer's instructions. The colorimetrical measurement was performed with a microplate reader (Infinite F200 PRO, TECAN) at a wavelength of 570 nm.

Based on the results of the above investigation on an optimal electroformed tubes design for stable dispensing, the inner diameter of an electroformed tube and the wall thickness were determined to be 0.5 mm and 50 μm, respectively. The tip angle of the electroformed tube was cut to 10° to ensure droplet attachment on the contact surface of a microplate well while maintaining dispensing precision. Moreover, preliminary experiment result showed that silicone oil was used to prevent the sample from drying. Additionally, the contact surface was coated with 0.1% poly-L-lysine solution before adding 5-μL silicone oil into each microplate well.

III. RESULTS AND DISCUSSION

In the present dispensing technique using an electroformed tube, we can strictly control the inner diameter of the tubes.

Figure 4 shows typical results of a cross-sectional image of electroformed tubes with an X-ray CT and a profile of the measured inner diameters. The most precise inner diameter was 499.0 ± 0.7 μm (Tube A). Since the inner diameter of the tubes can be freely controlled by selecting the outer diameter of an electroformed metallic wire, electroformed tubes with three kinds of inner diameter precision were prepared: less than 1 μm (Tube A), 10 μm (Tube B), and 30 μm (Tube C). The outer diameter of the plunger was 5 μm smaller than the measured inner diameter of the electroformed tube to maintain the ability to slide up and down while still holding liquid.

Table 1 shows the measurement results of the inner diameter precision of electroformed tubes and the results of the CV

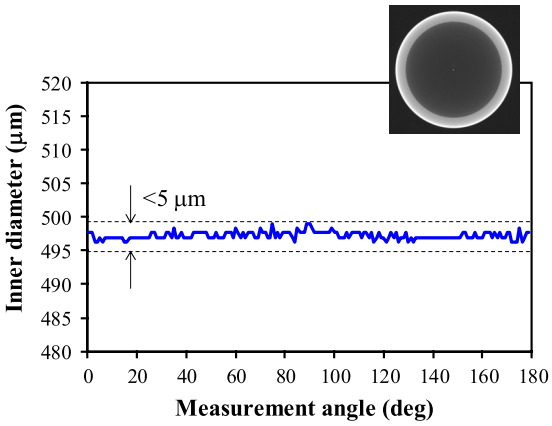

FIGURE 4. Measurement of inner diameter of 0.5-mm electroformed tube with high-resolution X-ray CT and example cross-sectional image.

TABLE 1. Relationship between inner diameter precision of three kinds of electroformed tubes and 100 nL dispensing precision.

	Inner diameter (μm)	CV value of dispensed volume (%) (N = 4)
Tube A	497.3 ± 0.6	1.55
Tube B	494.7 ± 9.2	3.08
Tube C	484.2 ± 28.6	4.70

in the case of dispensing 100 nL. The wall thickness and tip angle of the electroformed tube used was 50 μm and 10°, respectively with a hydrophobically treated surface. When comparing the three kinds of tubes with different inner diameter precision, it was found that dispensing precision improved as the inner diameter precision improved. In Tube A, with the best inner diameter precision of a manufacturing tolerance (±0.6 μm), the average CV value in dispensing 100 nL was 1.55%. On the other hand, in Tube C with inner diameter precision of a manufacturing tolerance of ±28.6 μm, the CV value was 4.70%. Since a positive-displacement technique was based on a constant-volume dispensing method, in which only the amount of solution pushed out by moving the plunger should be dispensed, it was considered that the precision of the inner diameter significantly affects the dispensing volume precision.

Figure 5 shows the dependence of dispensed volume precision on the wall thickness of the electroformed tubes. The wall thickness was set to 20, 50, or 80 μm. The surface was not hydrophobically treated, and the inner diameter and tip angle of the tube was fixed to 0.5 mm (plunger diameter of 0.495 mm) and 0°, respectively.

The CV values were changed from 0.96 to 2.90% (means, 2.25 ± 0.64) for a wall thickness of 20 μm, from 0.98 to 2.25% (means, 1.57 ± 0.28) for 50 μm, and from 1.53 to 4.19% (means, 2.82 ± 0.77) for 80 μm. The best averaged and most stable CV was achieved at a wall thickness

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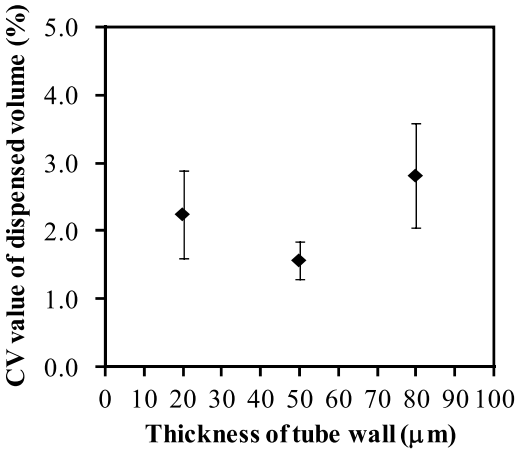

FIGURE 5. Dependence of dispensed volume precision on wall thickness of electroformed tubes. Values are expressed as mean ± standard error of mean (N = 3-5).

(a)

Wall thickness (μm)	20	50	80
A	539	462	447
B	202	195	192
C	455	394	363
D	368	403	467

(means, n = 6)

A: contact diameter of dispensing droplet
B: waist diameter of dispensing droplet
C: distance between contact surface and waist of dispensing droplet
D: distance between tube tip surface and waist of dispensing droplet

(b)

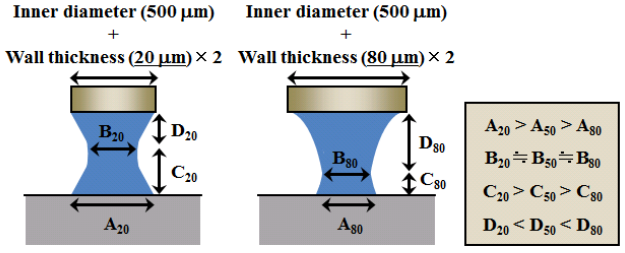

FIGURE 6. Analysis of dispensed droplet images obtained with high-speed camera.

of 50 μm. Based on these results, the following experiment was conducted with the wall thickness set to 50 μm.

The reason the CV values were most stable at a wall thickness of 50 μm is attributed to the stabilization of the tension of the dispensed droplet. Figure 6 shows the results of analyses of droplet dispensing images obtained with a high-speed camera. In Figure 6 (b), the relationships between each length (A to D) of a dispensed droplet and wall thickness are represented by letters with subscripted numbers. The real image was similar to that shown later (Fig. 8). The droplet waist (B) did not change greatly, but the droplet was pulled toward the tube tip side as the wall thickness increased. This is because the tube end face was not hydrophobically treated, so a thicker wall had a stronger force F $(=2\pi r\gamma)$ pulling the droplet by the outer periphery of the electroformed tube.

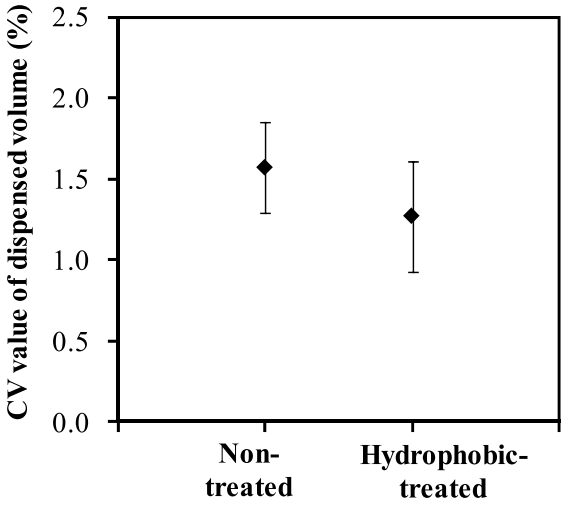

FIGURE 7. Dependence of precision of dispensed volume on surface hydrophobicity. Values are expressed as mean ± standard error of mean (N = 3-5).

Note that $2\pi r$ is the outer circumference of the tube, and γ is the surface tension of the liquid. For example, the surface tension of water (20°C) is constant at 72.75 mN/m.

The balancing forces acting on a dispensed droplet were gravity and the tension between the polystyrene surface and the force pulling the droplet by the outer periphery of the electroformed tube. When the upward and downward forces were balanced, a dispensed droplet would leave. Further studies are necessary, but within the scope of this study, it is thought that the equilibrium of the balancing forces is most stable when the wall thickness is 50 μm.

Figure 7 shows the dependence of the precision of the dispensed volume on surface hydrophobicity. The wall thickness was 50 μm. The inner diameter and tip angle of the tube were fixed to 0.5 mm (plunger diameter of 0.495 mm) and 0°, respectively. When focused on the CV values, it was from 0.98 to 2.25% (means, 1.57 ± 0.28) without hydrophobic treatment and from 0.70 to 1.88% (means, 1.27 ± 0.34) with hydrophobic treatment. When accuracy was calculated as reference data, the average value was 91.5% without surface treatment and 93.1% with hydrophobic treatment. The results showed that both the CV and accuracy values improved when the outside surface of the tube was hydrophobically treated. Thus, the best CV value for dispensing 100 nL was 0.7%, *i.e.*, 0.7 nL, which was significantly less than that observed for the current precision dispensers [11], [17]. Based on these results, the following experiment was conducted with hydrophobic treatment.

Figure 8 shows photos of the edge of an electroformed tube with and without hydrophobic treatment. A reduction in liquid retention was found in the vicinity of the outer periphery of the electroformed tubes (arrowheads in Figure 8), although it was not a significant difference. Previous reports also showed the feasibility of using the hydrophobicity of needle nozzles for dispensing microliter and nanoliter liquids [18], [19]. Thus, hydrophobically treating the outer surface of

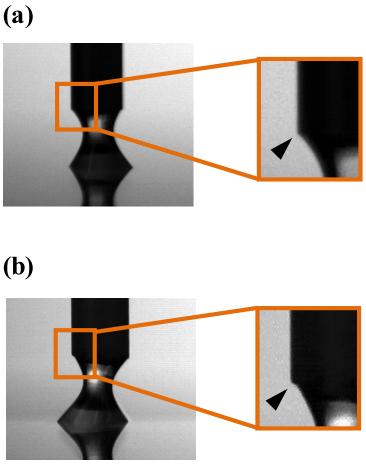

FIGURE 8. Photographs of the edge of an electroformed tube with (a) non-treated and (b) hydrophobic-treated.

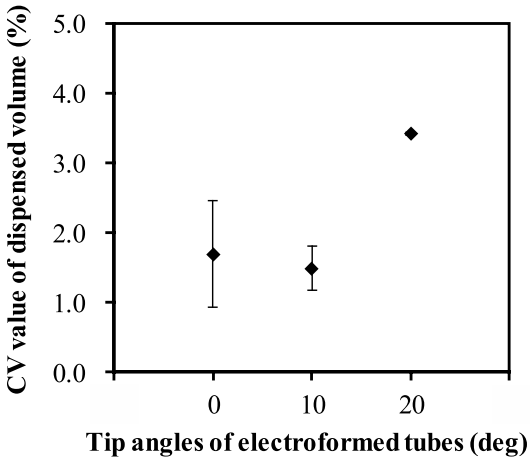

FIGURE 9. Dependence of electroformed tube tip angles on the CV of dispensing volume. Values are expressed as mean ± standard error of mean (N = 2-3).

an electroformed tube should enable smooth dispensing of the solution from the outer peripheral end of the tube. Our results showed that the CV values and accuracy should be better than those of a non-treated electroformed tube.

Figure 9 shows the dependence of dispensed volume precision on the tip angle of electroformed tubes. The tip angle of an electroformed tube was set to 0, 10, or 20°. The surface was hydrophobically treated, and the inner diameter of the tube was fixed to 0.5 mm (plunger diameter of 0.495 mm). The CV values were changed from 0.91 to 2.46% (means, 1.69 ± 1.09) for tip angle of 0°, from 0.94 to 2.04% (means, 1.49 ± 0.55) for 10°, and from 3.40 to 3.43% (means, 3.41 ± 0.02) for 20°. There was no big change at tip angles of 0° and 10°, CV <1% was achieved with the best value and the standard deviation was slightly smaller when the tip angle was 10°.

The oblique cylinder-shaped dispensing volume under an electroformed tube was 3.1 nL with a tip angle of 20° and 1.5 nL with 10°, respectively. A total dispensing volume was set to 100 nL, thus a surface tension for a droplet aside the electroformed tube would be stronger at tip angle of 20°.

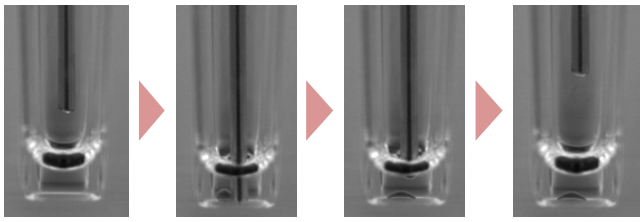

FIGURE 10. Serial photographs in dispensing process using an electroformed tube with tip angle of 10°.

Therefore, the volume variance could be larger. Based on a comparison of the averaged CV values, we chose the 10° tip angle. Further study to analyze force balances of a surface tension should be necessary, but we here used the 10° tip angle to demonstrate a feasibility of high-precision dispensing technique using a nickel electroformed tube.

Moreover, in the dispensing process, since a droplet is dispensed after an electroformed tube contacts the bottom surface of a microplate well, it is preferable that the tip angle is larger than 0° to avoid an unnecessary scratch on the surface. Figure 10 shows sequential photographs in the dispensing process onto a microplate well containing silicone oil to prevent sample drying. By using an electroformed tube with a tip angle of 10°, the droplet could be smoothly dispensed into a well while avoiding unnecessary contact with the tube edge to a microplate surface. Therefore, an electroformed tube with a tip angle of 10° was used in the following experiment.

To investigate a feasibility of highly reliable diagnosis of a limited volume blood sample, a glucose concentration in a dispensed 100-nL and 200- nL portion of animal-based control serum was measured colorimetrically with enzymatic reactions. The wall thickness of an electroformed tube used was 50 μm. The surface was hydrophobically treated, and the inner diameter and tip angle of the tube were fixed to 0.5 mm (plunger diameter of 0.495 mm) and 10°, respectively. After a drop of serum was dispensed without using drying or mixing reagents, the concentration of glucose was measured.

As shown in Figure 11, the CV value (n = 16) was 6.36% at 100 nL and 2.32% at 200 nL. In the biochemical analysis based on colorimetric measurements, the analytical precision was affected by not only sample dispensing precision but also conditions of enzymatic reagents and the reactions, the solution mixing, and photometric stability. Thus, some other variations were added to the dispensing precision, and the CV value of the blood analysis could be more degenerated than the above dispensing precision at 100 nL. However, these results suggest that several biochemical panels can be precisely measured even from one drop of blood.

In the technological translation into clinical application, there would be some concerns. Firstly, the estimated diagnosis cost will be either equivalent to or less than that of the conventional blood testing. Even if an electroformed tube could be relatively expensive, much less amounts of reaction reagents will be required for a micro-volume blood test. Second, as the expected difficulty for mass-production

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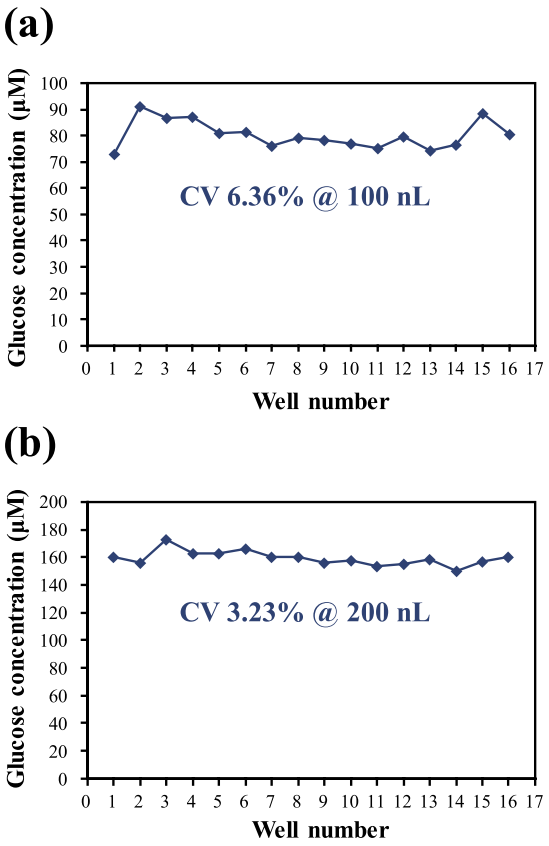

FIGURE 11. Results of glucose measurement in animal controlled serum.

of electroformed tubes, the individual difference should be considered. The effect on diagnosis results should be checked in a development phase of automated testing machine. Next, for the perspective of clinical application, an electroformed tube is quite safe since the dispensing device is not used for blood collection and the operators don't touch the dispensing tubes. Our electroformed tubes are made of nickel, therefore we should be careful for electrolyte testing if conducted in the future.

IV. CONCLUSION

We investigated the design of electroformed tubes for precise dispensing, and the results of testing showed that the optimal wall thickness is 50 μm. Furthermore, the outside surface should be hydrophobically treated to reduce water retention. The best coefficient of variation for 100-nL dispensing was 0.7%, *i.e.* 0.7 nL, which is significantly less than that observed in conventional precision dispensers. The stability of diagnosis with micro-volume serum was examined by measuring a glucose concentration in animal-based control serum that was dispensed using our positive-displacement dispensing system with an electroformed tube. The coefficient of variation was approximately 3% at 200 nL, suggesting that several biochemical panels can be precisely measured from just one drop of blood.

NOTE

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