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Dried Nanoparticle Label Reagents for Microfluidic Immunoassays

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Abstract— In this paper, we demonstrate and analyze the solubility and distribution of dried europium(III)-chelate dyed nanoparticles in mini channels of milled polystyrene (PS) cartridges. The particular objective for the study is to measure quantitatively the efficiency of the resuspension of dried nanoparticles from the surface of PS channels and to determine the homogeneity of the solute particles in a reaction chamber considering also the incubation time.

Keywords- dry chemistry; homogeneity; immunoassay; nanoparticle; passive valve; solubility

I. INTRODUCTION

Immunoassays have been used for decades in clinical chemistry and nowadays they cover 40% to 50% of the *in vitro* -diagnostics industry [1]. However, still many assays are done only in the central laboratories. Point-of-care testing (POCT) [2] and lab-on-a-chip (LOC) [3, 4] applications have gained their interest in the diagnostics due to the benefits and better know-how of miniaturization [5] and microfluidics [6]. In the LOC applications there are challenges for the integration of microfluidics: pumps [7], valves [8], mixing [9], etc. In spite of the investments for the research and development in this field only few successful commercial POCT and LOC products are in the market.

In the traditional immunoassay techniques, the liquid assay reagents (wet-chemistry) are manually dosed into open cuvettes (e.g. well plates). The trend for LOC and POCT platforms will push the development of immunoassay methods to integrate dry-chemistry storage into the chip. Drying the reagents in a chip instead of using wet-chemistry provides longer-term storage, decrease of expensive reagent waste and makes the devices more portable and robust. However, the topic is still relatively unexplored and only very few publications exist.

Applications with integrated dry reagent membranes in a chip have been presented in [10, 11]. In this approach, an efficient release of the dry reagents from the membrane might be challenging because particles tend to adhere on the surfaces through non-specific interactions. In applications, where dried substances are preserved on a channel [12] or

on a reaction well [13], the unspecific binding is reduced due to the reduction of surface to volume ratio. However, quantitative studies on the solubility of dried particles have not been reported.

Time-resolved fluorometry and lanthanide chelate labels embedded in particles of approximately 50 - 100 nm in diameter offer an extremely sensitive detection system for the immunoassays. In this study, antibody-coated europium (Eu)-containing nanoparticles [14] were used.

The particular objectives for the study presented here are to measure quantitatively the release of dried nanoparticles from polystyrene (PS) mini channels and to determine the distribution of the solute particles in a reaction chamber using time-resolved fluorescent detection.

The rest of the paper is arranged as follows. Section 2 describes the experimental setup, materials, and methods. Section 3 presents the results of the measurements and Section 4 provides the analysis of the results. Finally, Section 5 concludes the paper.

II. MATERIALS AND METHODS

A. Fabrication of Cartridges

The cartridges used in these experiments were fabricated using mechanical milling on a 3mm thick transparent PS plate as shown in Fig. 1A and 1B. The cartridge consists of the following main parts: buffer input, main channel, reaction chamber, passive valve, and pressure connection. The cross-sectional shape of the channel is a half circle having a diameter of 1,5mm. The volume of the reaction chamber, where fluorescent excitation finally takes place, is 40 μ l and has a flat bottom surface to ensure homogeneity in the reaction area.

B. Dry Chemistry in the Cartridge

The nanoparticles used in this study were Eu-dyed particles of 92 nm in diameter from Seradyn (Indianapolis, USA). The particles were covalently coated with recombinant Fab-fragments of estradiol-specific antibody S16 as described earlier [14]. Approximately 100 active Fab-fragments were obtained per one particle. The particles are intended for proximity-based homogeneous immunoassays

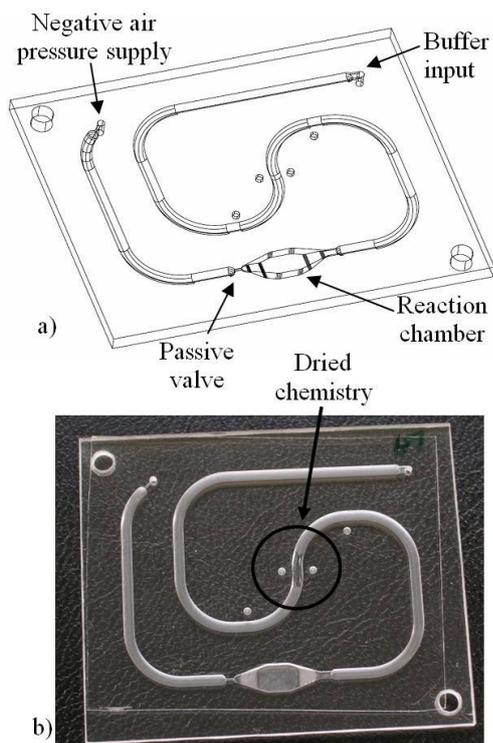


Figure 1: A) Milled polystyrene cartridge design for the experiments. B) Dried Fab-coated Eu-nanoparticles

of estradiol (E2, female sex hormone). This study focused on surveying the conditions for dissolving dried particles from the channels, and therefore, direct measurement of particles was used instead of a complete E2-assay as a measure of resuspension degree.

The Fab-coated particles were diluted in a buffer containing the following composition: 37.5 mM Tris-HCl, pH 7.75, 120 mM NaCl, 0.375 g/l NaN_3 , 0.6 g/l bovine- γ -globulin, 25 g/l bovine serum albumin, 50 g/l trehalose, 0.1 g/l native mouse IgG, 0.05 g/l denatured mouse IgG, and 2 g/l casein. Three different sets of volumes ($V_A=1\mu\text{l}$, $V_B=3.4\mu\text{l}$ and $V_C=7.5\mu\text{l}$) of the nanoparticle solution were manually dispensed on a specific place in the middle of the milled flow channel (Fig. 1B). Solutions included 6×10^7 ($V_A=1\mu\text{l}$), 20.4×10^7 ($V_B=3.4\mu\text{l}$), and 9×10^7 ($V_C=7.5\mu\text{l}$) particles and are further named as Series A, B, and C, respectively. After the nanoparticles with appropriate chemistry were dried over night, the cartridges were covered using adhesive tape for molecular diagnostics from Adhesives Research type ARseal™ DEV-90404. Altogether ten pieces of cartridges for each Series were prepared for the tests.

C. Measurement Procedure

The user manually dispensed $44\mu\text{l}$ of assay buffer (Innotrac buffer clear, Inntrac Diagnostics Oy, Turku, Finland) to the cartridge via the buffer input using a pipette. A high precision computer controlled electro-pneumatic transducer unit (own development) was connected to the negative pressure supply of the test cartridge using a short

silicone tube and a standard Eppendorf pipette tip. Two different aspiration pressures were generated. The lower control pressure was -14mbar and higher -24mbar, indicated later as Slow Flow and Fast Flow, respectively. Negative pressure was used to drive the solution into the reaction chamber until stopped by the passive valve in a cartridge. The flow control was performed utilizing a LabVIEW based program.

Time count was started when the assay buffer touched the dried zone very first time (indicated as Starting Time). The buffer was driven into the reaction chamber using constant aspiration pressure. Thereafter, the pressure connection was released and the cartridge was placed on a plastic positioner, which guaranteed the right position in the Victor² plate reader (PerkinElmer Life Sciences, Wallac Oy, Turku, Finland) where fluorescent analysis took place. The fluorescent measurement sequence started promptly in 2 min from Starting Time.

A scanning property of the reader was used to measure a "local" number of particles in the reaction chamber of the cartridge. The scanning matrix included 15 measurement points and covered the area of $4\text{mm} \times 2\text{mm}$, as shown in Fig. 2.

The measurement sequence was started promptly 2min from the Starting Time, then incubated for 3,5min and measured again, and again incubated for 3,5min followed by the last measurement. The total time for one procedure was 10,5min. The measurements are indicated as 2min, 6min, and 10min incubation times.

Altogether 30 measurements with dried Fab-coated Eu-nanoparticles were performed. The measurement procedure was repeated five times for each of combination of the three different volumes of dry chemistries and the two different flow speeds.

Reference data was measured using cartridges without dried reagents. A reference solution contained the identical number of Eu-nanoparticles (6×10^7 , 20.4×10^7 , and 9×10^7 as in Series A, B, and C, respectively) with those dried on the other cartridges. $44\mu\text{l}$ reference solution was pipetted into the channel and driven through the flow channel to the reaction chamber using Slow Flow. Fluorescent counts for references were measured after 2min, 6min, and 10min incubation. These values provide the "100 % reference level" for calculations and comparisons to the other experiments.

In the reference measurement, the nanoparticles stayed in the channel a longer distance than in the tests with dried particles. Some of the particles may have adhered to the channel walls from the solution, and therefore, the effect of

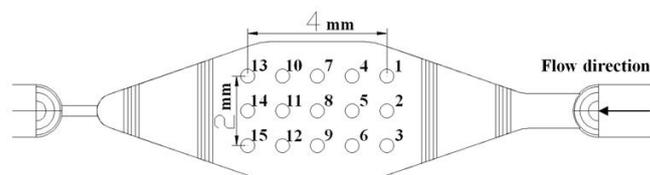


Figure 2: 5x3 scanning matrix of the reaction chamber

unequal distances should be compensated. However, adhesion from the solution is considered so slight that in this research and analysis the effect is ignored.

III. RESULTS AND DISCUSSIONS

The numbers of particles in the three different Series (A, B, and C) were equalized for calculations to make comparison possible between the Series. All data were equalized to 1million particles so that all data for A-Series was divided by 60, for B-Series was divided by 204, and for C-Series was divided by 90.

Background noise for Europium fluorescence was measured in the Victor² plate reader using an empty cartridge and it was not significant comparing to the other experiment values.

Here we present the results of the measurements for resuspension of dried nanoparticles and distribution of the solute particles in the reaction chamber. The parameters are named as Solubility Number (SN) and relative Homogeneity Number (rHN). Also the effect of the incubation time on the homogeneity and the deviation in rHN between replicated cartridges are presented.

A. Solubility of Dried Nanoparticles

Solubility Number (SN) describes how efficient is the solubility of Eu-nanoparticles to the buffer solution. SN was calculated using (1), which is the mean value of the measurements collected from the 15 measurement spots of the scanning matrix. The larger the SN, the larger number of particles there are in the reaction chamber and the better is the solubility of the particles.

$$SN = \frac{1}{15} \sum_{i=1}^{15} a_i, \quad (1)$$

where a_i is a number of photon counts of a single spot i ($=1...15$) in the scanning matrix in Victor² plate reader. The efficiency of solubility is determined as a ratio between the SNs (for each Series) and the reference SN.

Table 1 presents percentage values of SN ratios for each Series. In this comparison, the SN values are average from the five replicate cartridges having the same dry volume and flow speed. SN data in the table was collected after 10min incubation.

Comparing the relative solubility numbers, the efficiency of solubility is generally higher with Slow Flow when the buffer solution has more time to wash out the particles from the channel surface. A lower drying volume is also better for solubility when there are not so many nanoparticles to dissolve. Incubation does not change dramatically the Solubility Numbers but, in general, lowers them slightly, as shown in Fig. 3A.

TABLE I. SOLUBILITY NUMBER (SN) AND RELATIVE HOMOGENEITY NUMBER (rHN) FOR REFERENCE AND FOR THE EXPERIMENT SETS AFTER 10MIN INCUBATION

	Series		
	A	B	C
Dried Volume	1 μl	3,4 μl	7,5 μl
<i>Ref. SN</i>	21788	22424	23883
<i>SN and Solubility % (Fast Flow)</i>	8155 (37%)	3505 (16%)	3281 (14%)
<i>SN and Solubility % (Slow Flow)</i>	21704 (100 %)	16105 (72%)	14737 (62%)
<i>Ref. rHN</i>	0.022	0.051	0.032
<i>rHN (Fast Flow)</i>	0.228	0.353	0.312
<i>rHN (Slow Flow)</i>	0.381	0.208	0.114

B. Relative Distribution of the Solute Chemistry in Reaction Chamber

The relative distribution in the reaction chamber is described numerically as a relative Homogeneity Number (rHN), as shown in (2). The lower the rHN the more homogeneous is the distribution of the nanoparticles in the reaction chamber.

$$rHN = \frac{\sqrt{\frac{1}{15} \sum_{i=1}^{15} (a_i - SN)^2}}{SN}. \quad (2)$$

where a_i is a number of photon counts of a single spot i ($=1...15$) in the scanning matrix in Victor² plate reader. SN is Solubility Number.

In Table 1, the rHNs are compared to known reference values calculated using (2). In this comparison, the rHN values are an average from the five repeats of the data, collected after 10min incubation. The relative Homogeneity Number is highest (0.381) with low volume of dry chemistry using Slow Flow. Although the solubility of the particles in this case was 100% the homogeneity was very poor. The smallest rHN (0.114) occurs when using high volume of dry chemistry and Slow Flow to achieve relatively good solubility and homogeneity.

C. Incubation-time Dependent Distribution of the Particles

Fig. 4 presents a typical distribution of resuspended particles in the reaction chamber during increasing incubation time. The Solubility Numbers (referring to the amount of solute particles in the chamber) are expected to equal at various time points. The slight decrease observed in the SN-values, as shown in Fig. 3A, may be due to mild bleaching of the label after intense measurements (though Eu-particles typically are quite tolerant to repeated measurements).

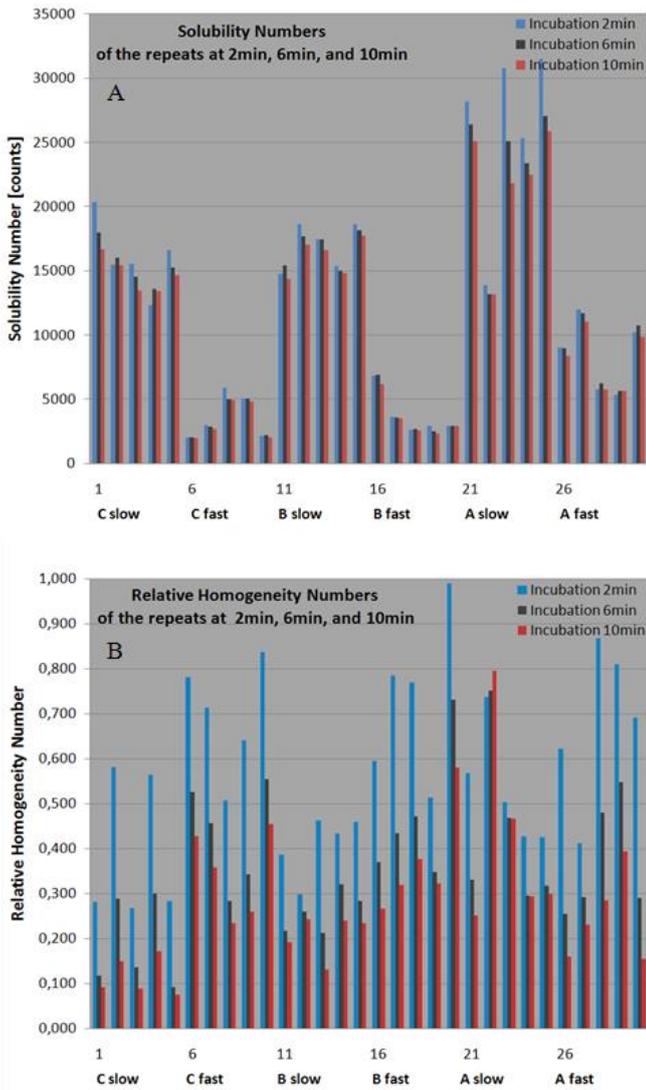


Figure 3: A) Solubility Numbers (SN) and B) relative Homogeneity Number (rHN) after 2min, 6min, and 10min incubation for all 30 measurements

Incubation time affects dramatically to homogeneity of the solute particles in a reaction chamber as presented in Fig. 3B. In all cases, the distribution of the particles in the chamber is very uneven at the beginning of experiment. When incubated, the particles diffuse over the reaction chamber and the distribution becomes more even as Fig. 4 illustrates.

D. Deviation between Cartridges

Deviation of the results between different cartridges was compared calculating the rHN for a single measurement spot (rHN_{spot}) in replicate cartridges having the same dry volume and flow speed, as shown in (3). This describes the variations between the single measurement spot. One set includes five replicate cartridges measured with the same parameters, and rHN_{spot} is calculated between those data points.

$$rHN_{spot}(i) = \frac{STD_{spot}}{AVG_{spot}} = \frac{\sqrt{\frac{1}{5} \sum_{j=1}^5 \left(a_{ij} - \left(\frac{1}{5} \sum_{j=1}^5 a_{ij} \right) \right)^2}}{\frac{1}{5} \sum_{j=1}^5 a_{ij}} \quad (3)$$

where a_{ij} is a number of photon counts of a certain spot i ($=1 \dots 15$) in the scanning matrix and j ($=1 \dots 5$) represents the different replicate cartridges.

It can be seen from Fig. 5 and Fig. 6 that there is a huge variation at the beginning of each experiment but after incubation the variations between single spots in different cartridges become closer to each other. Fig. 5 presents all calculated rHN_{spot} values for each spot with all three incubation times. It can be seen that the biggest variation occurs in the back of the reaction chamber. But during the incubation deviation becomes smaller. In Fig. 6 the effect of the incubation is shown and averages of the rHN_{spot} values of the one cartridge set with different incubation times are compared. The smallest variations occur in series C with Slow Flow speed and after 10min incubation.

E. Analysis of the Results

This chapter gathers the results presented in previous chapters, where the Solubility Number describing solubility of dried nanoparticles and relative Homogeneity Number describing the relative distribution of the solute chemistry in the reaction chamber were calculated. Also the effect of incubation and the deviation between cartridges were determined.

The results show that typically the most intensive fluorescent signals are collected at the beginning of the reaction chamber. The particle distribution in the reaction chamber is at the beginning of the incubation very uneven but become even during the incubation. The best solubility results were achieved with Slow Flow rate and it was slightly higher for small volumes ($1\mu l$) of dried chemistry than for the larger volumes. If the flow speed is too fast dried nanoparticles will not dissolve into the passing liquid.

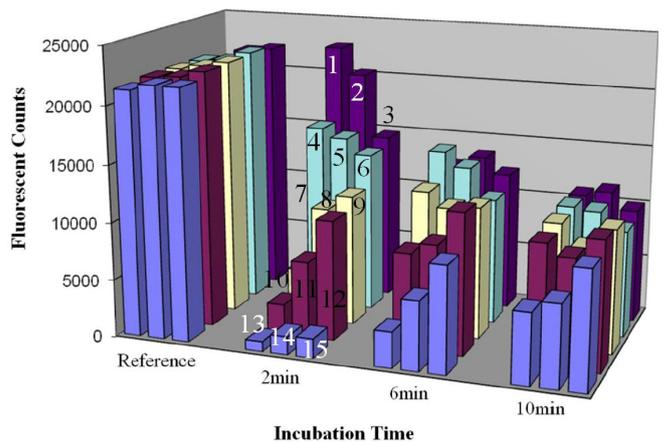


Figure 4: Fluorescent counts of scanning matrix spots (1 to 15) and effect of incubation time for particle distribution in a reaction chamber

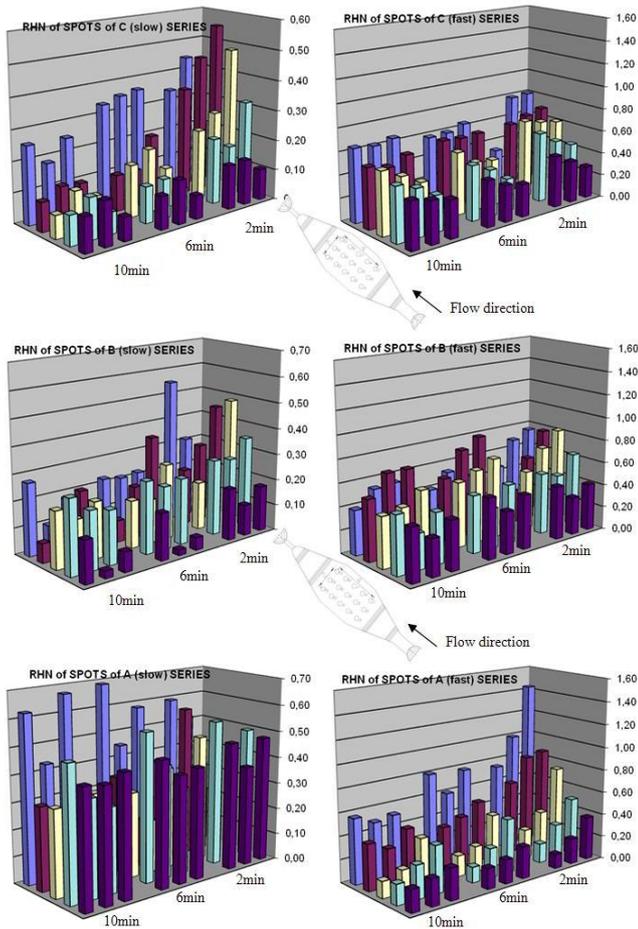


Figure 5: Relative Homogeneity Numbers calculated for each measurement spots for each Series

Even though small volumes and slow flow rates gave good results for the solubility, the rHN values for that combination were not as good. The values in Table 1 show that using high volume (7.5 μ l) of dry chemistry and Slow Flow speed the solubility is relatively high and homogeneity in the chamber is very good the rHN value being the best (0.114).

Incubation time dependent effect on the particle distribution in the reaction chamber is remarkable. Incubation time does not affect so much the SN values but when incubated the particles diffuse evenly over to the reaction chamber improving the rHN values, as shown in Fig. 4. The results show that a considerable incubation time is needed to guarantee uniform particle density in the reaction chamber. This is an important aspect to perceive when designing assays in the cartridge.

The deviation between replicated cartridges is large with short incubation times, as shown in Fig. 5. Larger incubation times decrease the variation as well as slow flow rates and large dry volumes. Therefore, a higher repeatability in the microchannel assays with dried nanoparticles can be achieved using slow flow speeds, large dry volumes and long incubation times.

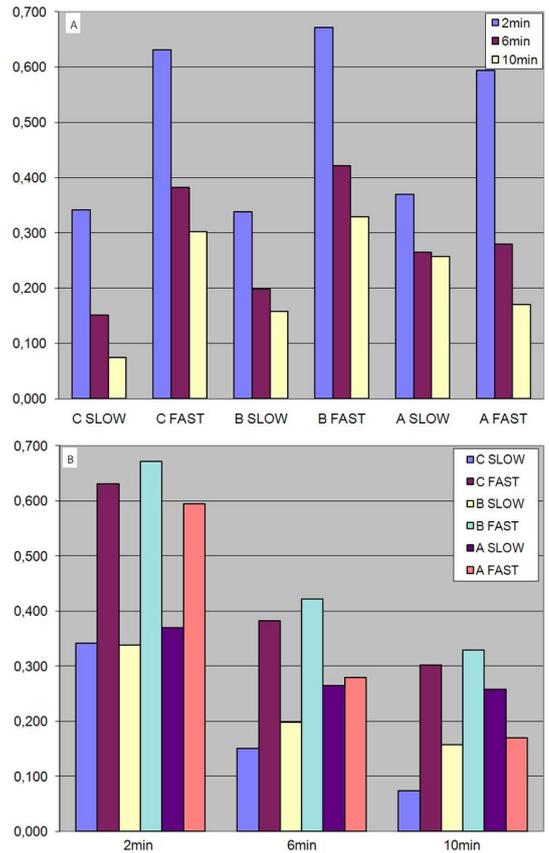


Figure 6: A) Effect of incubation time to the average of rHN_{spot} of replicate cartridges B) Comparison of averages of rHN_{spot} of replicate cartridges at various time points

IV. CONCLUSION

We explained and analyzed the solubility and distribution experiments of dried Europium-labelled nanoparticles in small milled polystyrene cartridge channels. The goal was to measure the efficiency of the release of the nanoparticles from the surface of the channel and to specify the distribution of the particles in the reaction chamber of the cartridge.

Tests were performed utilizing pressure driven flow control over the dried particles to drive the solution into the reaction chamber. Measurements were done using Victor² plate reader to detect fluorescent emission of Eu-labels. A spot matrix (5x3) was used to scan the entire chamber three times during the 10min incubation. 3 different drying volumes (1 μ l, 3.4 μ l, and 7.5 μ l) were used with 2 different overflow speeds (constant pressure). The data was collected and analyzed.

The analysis clearly shows that the best choice in this type and size channels is to use dry volumes around 5 μ l to 7.5 μ l and to use slow flow speed to dissolve the dry chemistry. That combination achieves a good level of solubility (around 70% from reference) and a good level of homogeneity of the particles in the reaction chamber in an appropriate time. It is also shown that at least 10min incubation is needed for an even particle distribution in the

chamber. The uneven distribution observed at shorter incubation times is a remarkable source of variation and therefore, it would impair the repeatability of replicate immunoassays made in the cartridge.

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