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# A spectrophotometric procedure for DNA assay with a micro-sequential injection lab-on-valve meso-fluidic system

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**Abstract**

A micro-sequential injection spectrophotometric procedure for DNA assay was developed based on the employment of a lab-on-valve (LOV) meso-fluidic analytical system. A small amount of crystal violet solution (10 μl) was de-colored inside the flow cell of the LOV at the presence of 5 μl λ-DNA/*Hin*dIII within a certain pH range, and the absorbance decrease of crystal violet solution at 591 nm was measured via optical fibers and was employed as the basis of quantification. A uni-variant approach was adopted for the optimization of experimental parameters, including buffer pH, concentration and volume of crystal violet solution, reaction time and sample/reagent loading flow rates. A linear calibration graph was obtained within 0.2–6.0 μg $ml^{-1}$, along with a detection limit of 0.07 μg $ml^{-1}$. The procedure was applied for the determination of λ-DNA/*Hin*dIII in synthetic samples in comparison with a documented procedure.


*Keywords:* Sequential injection; Lab-on-valve; Meso-fluidic analytical system; Spectrophotometry; DNA assay

## 1. Introduction

Quantification of small amounts of DNA has gained extensive attention in a wide variety of biological applications, e.g., determination of the yields of purified DNA fragments for sub-cloning and the yields from cDNA library production. For genetic diagnosis, the assay of DNA amplification products or DNA contamination arising from recombinant organisms in drug preparations is frequently called for. Furthermore, for forensic analysis, rapid and sensitive quantification of small amounts of DNA obtained either by direct extraction or by amplification from blood, semen, bone, or other sources is a critical step prior to genotyping.

A variety of analytical procedures for DNA assay have been developed, including spectrophotometry [1–4], fluorimetry [5–8], chemiluminescence [9,10], light-scattering [11,12] and electrochemical techniques [13–15]. Among these protocols, spectrophotometry is playing an important role owing to its simplicity, good reliability/reproducibility and cheap instrumentation. To our best knowledge, the majority of procedures of this category were developed based on the decoloration of certain dyes in the presence of DNAs, and proper selectivity can usually be achieved due to the specific binding between nucleic acid and the dye. When operated in batch mode, however, they are time consuming and labor intensive, and, most critically, the expense of large amount of sample and reagent is non-compatible for bioassays [2,3]. At this point, automated on-line procedures with minimized rare samples and/or expensive reagents consumption are highly demanded for DNA assays. Literature search located, however, only a single article regarding flow injection assay based on the decoloration reaction of methyl violet in the presence of fish sperm DNA [16]. The detection limit of this procedure cannot fulfill the requirements of most analytical purposes, in addition, sample/reagent consumption goes far beyond the acceptable range of routine bioassays. At this juncture, micro-sequential injection system incorporating a lab-on-valve (LOV) unit has been proven to be a superb alternative for downscaling sample pretreatment [17–19], since its first commencement [20]. A conception of meso-fluidic analytical system was recently proposed [21,22] based on the em-

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ployment of a lab-on-valve unit, by which means 0.1–10 μl fluids can readily be manipulated precisely. This is especially useful for bioassays based on microliter levels consumption of rare/expensive reagent/sample.

In the present communication, we report an on-line spectrophotometric procedure for the determination of DNA with a meso-fluidic analytical system. The procedure was developed based on the decoloration reaction of crystal violet solution in the presence of DNA [4]. The crystal violet is among a group of dyes that shows a color change on binding to poly-anions such as nucleic acids at low concentration. This color change is unusual in that the dye exhibits a substantial decrease in absorbance at the wavelength of maximum absorption on binding to nucleic acids, thus, imparting a color difference from the dye itself on the stained substance, this effect is known in histology as metachromatic staining [1,23]. The absorbance change is proportional to the concentration of DNA in a certain range, and thus, settled the basis of quantification. The employment of a lab-on-valve unit not only minimized sample processing and reagent consumption, but also facilitated in situ real time monitoring of the reaction process.

## 2. Experimental

### 2.1. Instrumentation

Experiments were performed by employing a FIAlab 3000 sequential injection system (FIAlab Instruments Inc., Bellevueo, WA, USA) equipped with a 2.5 ml syringe pump and a lab-on-valve unit (FIAlab Instruments Inc.). As illustrated in Fig. 1, the LOV integrates all the flow channels and sampling ports, in which various reagent-based assays can be performed by varying the experimental protocols. A multipurpose flow-through cell, with a volume of about 20 μl, is permanently incorporated into port #2, which allows a series of miniaturized fluidic operations to be performed, including reagent addition, dilution, mixing and incubation. The flow cell is furnished with 400 μm fiber optic cables (OceanOptics Inc., Dunedin, FL, USA), communicating with an external light source and a spectrophotometer. The incident light from a tungsten halogen lamp (OceanOptics Inc.) was carried to the flow cell, while the transmitting light was directed to a USB2000 spectrophotometer (OceanOptics Inc.) for measuring the absorbance. The entire system was controlled with a personal computer by running the FIAlab software for Windows, version 5.9.163.

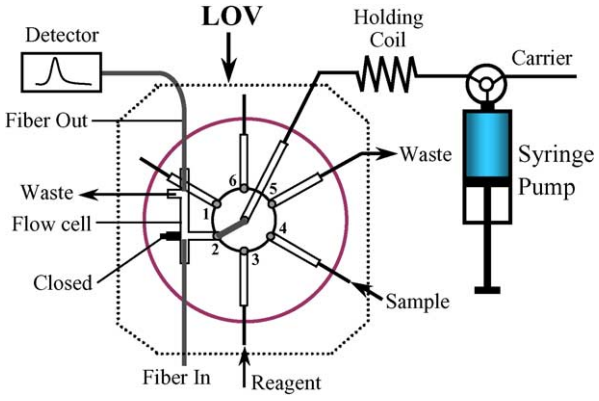


Fig. 1. Flow manifold of the micro-sequential injection lab-on-valve mesofluidic system for DNA quantification with spectrophotometry.

### 2.2. Reagents

All the reagents used were at least of analytical grade, and 18 MΩ ion-free water was used throughout.

A $1.0 \times 10^{-3}$ mol l$^{-1}$ crystal violet (Disan Chemicals Co., Shenyang) stock solution was prepared by dissolving 0.0570 g crystal violet in ion-free water and further diluted to 100 ml; working solutions of different concentrations were obtained by step-wise dilution of the stock solution.

A $5.0 \times 10^{-2}$ mol l$^{-1}$ Tris–HCl buffer solution (pH 8.6) was prepared by mixing 50 ml 0.1 mol l$^{-1}$ Tris solution (Merck, Darmstadt) with 12.4 ml 0.1 mol l$^{-1}$ HCl (Shenyang Chemicals Co., Shenyang) and the mixture was diluted to 100 ml with ion-free water.

λ-DNA/*Hin*dIII was purchased from Huamei Biological Engineering Co., Luoyang, and working solutions of different concentrations were further diluted with Tris–HCl buffer (50 mM Tris, pH = 8.6).

### 2.3. The operating procedure

Two milliliter water and appropriate amount of sample and crystal violet solutions were first sequentially aspirated into the holding coil, where the various zones are stacked. Thereafter, the syringe pump is immediately set to propel the stacked sample/reagent zones forward at a flow rate of 7 μl s$^{-1}$. The adjacent zones dispersed into each other, and thus, allowed the de-coloration of the crystal violet solution to take place on their way flowing toward the flow-through cell. The absorbance of the crystal violet solution in the dispersed zone in the presence of DNA ($A$) was measured at 591 nm against water in the flow cell, and the absorbance ($A_o$) of the corresponding reagent blank in the absence of DNA was measured under identical conditions. A calibration graph was, thus, developed based on the relationship between the absorbance differences, $\Delta A$ ($A_o - A$) and the concentration of DNA.

## 3. Results and discussion

### 3.1. Optimization of the experimental variables

#### 3.1.1. The pH of buffer solution

Tris–HCl buffer solution was used to dilute the DNA containing sample solution. The experiments indicated that the pH of this buffer solution has significant effect on the decol-

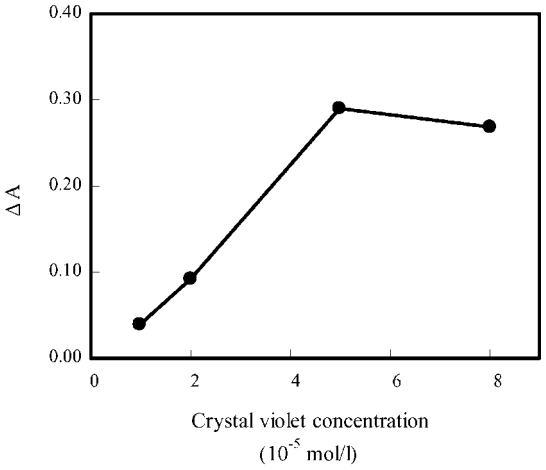


Fig. 2. The effect of crystal violet concentration. Volume of crystal violet: 10 μl; concentration and volume of λ-DNA/*Hin*dIII: 5 μg ml$^{-1}$, 5 μl; pH = 8.6; sample loading flow rate: 7 μl s$^{-1}$.

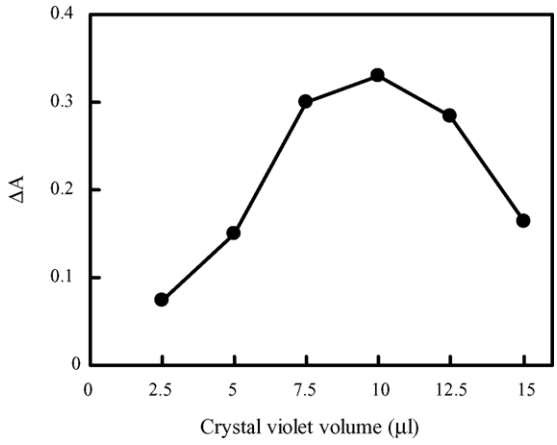


Fig. 3. The effect of crystal violet volume. Concentration of crystal violet: $5 \times 10^{-5}$ mol l$^{-1}$; concentration and volume of λ-DNA/*Hin*dIII: 5 μg ml$^{-1}$, 5 μl; pH = 8.6; sample loading flow rate: 7 μl s$^{-1}$.

oration reaction of crystal violet solution in the presence of DNA and the decoloration increased substantially with the increase of pH, i.e., the absorbance difference, $\Delta A$, was increased rapidly as the pH of the buffer increased from 7.2 to 8.6, while afterwards, a further increase of the buffer pH resulted in a slight drop of the absorbance difference at pH 9.0. A buffer pH of 8.6 is, therefore, employed for the ensuing experiments.

### 3.1.2. The concentration and volume of crystal violet

For spectrophotometric procedures based on decoloration reaction, it is critical to set the concentration of the color reagent within an appropriate range in order to obtain an optimal sensitivity. Fig. 2 illustrated the influence of the concentration of crystal violet solution within $1 \times 10^{-5}$ to $1 \times 10^{-4}$ mol l$^{-1}$ in the presence of 5 μl λ-DNA/*Hin*dIII solution (5 μg ml$^{-1}$). It can be seen that the absorbance difference obtained increased steeply with the increase of crystal violet concentration up to $5 \times 10^{-5}$ mol l$^{-1}$, while afterwards a decline was recorded by further increasing its concentration. A possible explanation for the observed phenomena is that when a lower concentration is employed, a slow decoloration reaction of the crystal violet solution in the presence of DNA (forward reaction) is recorded. The increase of crystal violet concentration in a certain range resulted in an increase of the forward reaction rate, and thus, the recorded absorbance difference. At the same time, the concentration of the decolored product is increased, which in turn accelerated the reverse reaction and restrained the forward reaction. Fig. 2 showed that the curve was almost levelled off when crystal violet concentration exceeded $5 \times 10^{-5}$ mol l$^{-1}$, which might be an indication of the establishment of an equilibrium. In order to maintain a reasonable sensitivity of the determination, a concentration of $5 \times 10^{-5}$ mol l$^{-1}$ for the crystal violet solution was, thus, selected for further investigations.

In the present set-up of the LOV system, the decoloration is mainly affected by dispersion of the stacked crystal violet and DNA sample zones; it is, thus, critical to utilize an appropriate volume of reagent zone. Fig. 3 showed the relationship between the readout and the volume of crystal violet solution within a range of 2.5–15 μl, by fixing the volume of DNA solution at 5 μl. An obvious increase in the absorbance change was observed as the volume was increased from 2.5 to 10 μl, thereafter, a decline of the signal was recorded by further increasing the volume up to 15 μl. The reason is apparent that within the time span of the reaction, the adjacent sample/reagent zones in the channel disperse into each other, and thus, allowing the reaction to take place and give rise to the decolored product, the amount of which was increased with the increase of reagent zone volume within a certain range, thus, an increase of the absorbance difference was observed. When an excessive reagent zone was introduced into the flow cell, however, the DNA sample zone can only disperse/penetrate into a certain length of it, thus, leaving a part of the zone with pure reagent. This part of the zone dominated the recorded absorbance, i.e., the absorbance arisen from the decolored product was merged, as a result, a drop of the absorbance change with further increase of the crystal violet zone volume was obtained. For the ensuing studies, a 10 μl of crystal violet zone was, therefore, employed.

### 3.1.3. The reaction time and flow rate

Various reaction times were selected in order to investigate the decoloration rate. Fig. 4 illustrated the recorded results with stopped flow technique. It is obvious that the observed absorbance difference dropped rapidly with the increase of residence time of the sample/reagent zone. This indicated that the decoloration of crystal violet in the presence of DNA is a fast reaction, which appears to be completed immediately. The dispersion effect might result in a decline of the signal, and thus, the sensitivity. It is, therefore, beneficial to transport the reaction mixture rapidly into the flow-through cell for tracking the reaction process, and no stopped flow is em-

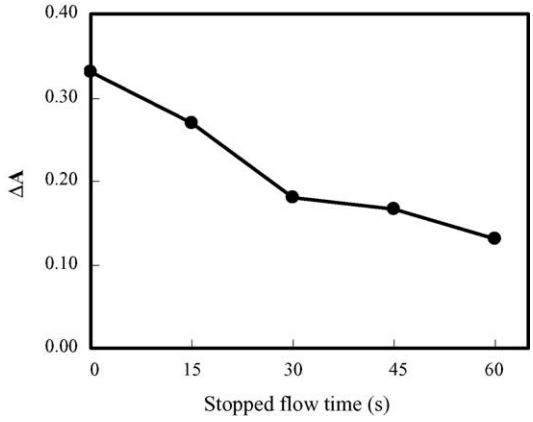


Fig. 4. The effect of reaction time/stopped flow time. Concentration and volume of crystal violet: $5 \times 10^{-5}$ mol l$^{-1}$, 10 μl; concentration and volume of λ-DNA/*Hin*dIII: 5 μg ml$^{-1}$, 5 μl; pH: 8.6; sample loading flow rate: 7 μl s$^{-1}$.

ployed. In order to carry the reaction mixture into the flow cell immediately, it seems that a faster flow is preferential. The effect of flow rate on the absorbance difference was investigated in the range of 2–10 μl s$^{-1}$, as illustrated in Fig. 5. It can be seen that the readout was indeed increased with the increase of flow rate below 7 μl s$^{-1}$, while afterwards the recorded signal dropped down when even higher a flow rate was employed. For the ensuing experiments, a loading flow rate of 7 μl s$^{-1}$ was selected.

### 3.1.4. The ionic strength and salt effect

It has been demonstrated that ionic strength can significantly affect the decoloration of chromogenic reagents in the presence of DNA. It is, thus, mandatory to maintain the ionic strength of the reaction system not to exceed a certain range, in order to obtain a reasonable sensitivity, and at the same time to minimize salt effect. This was exploited by adding various amount of NaCl into the DNA containing sample, Fig. 6 illustrated its effect both on the absorbance of the crystal violet solution and the decolored product. The results indicated that

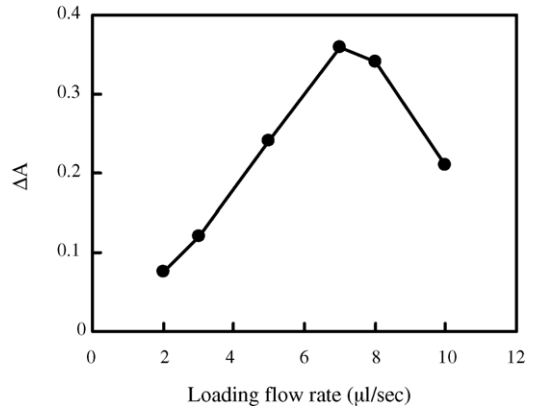


Fig. 5. The effect of sample/reagent loading flow rate. Concentration and volume of crystal violet: $5 \times 10^{-5}$ mol l$^{-1}$, 10 μl; concentration and volume of λ-DNA/*Hin*dIII: 5 μg ml$^{-1}$, 5 μl; pH: 8.6.

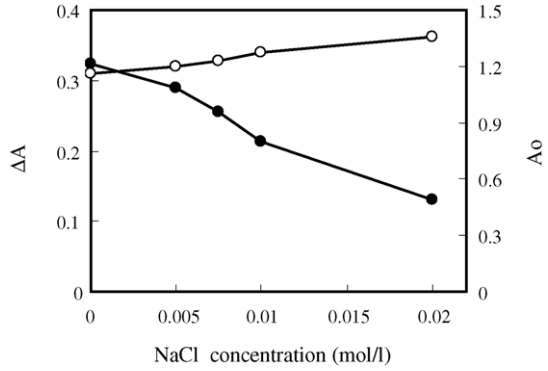


Fig. 6. The effect of ionic strength (NaCl concentration). Concentration and volume of crystal violet: $5 \times 10^{-5}$ mol l$^{-1}$, 10 μl; concentration and volume of λ-DNA/*Hin*dIII: 5 μg ml$^{-1}$, 5 μl; pH: 8.6; sample loading flow rate: 7 μl s$^{-1}$. (○) Absorbance and (●) absorbance difference.

the effect is well controlled when the concentration of NaCl is no more than $5 \times 10^{-3}$ mol l$^{-1}$.

### 3.2. Interfering effects

Considering that the matrix compositions are usually quite complex in biological samples, i.e., various components in biological matrix might cause interferences for the assay of DNAs. The potential interfering effects of some of these species frequently encountered in biological samples were, thus, investigated. The experimental results indicated that for the assay of 5 μg ml$^{-1}$ λ-DNA/*Hin*dIII, no interfering effects were observed arising from alkaline earth and heavy metals at the concentration levels found in body fluids, within a ±5% error range. The effect of NaCl concentration is, however, quite pronounced when exceeding 0.01 mol l$^{-1}$ as illustrated in Fig. 6. Considering that blood and body fluids might contain up to 0.1 mol l$^{-1}$ NaCl, it is, thus, necessary to incorporate a sample pretreatment procedure, e.g., appropriate dilution could effectively attenuate the matrix effects. On-line isolation of DNA from the matrix components should be the most effective approach. A solid phase extraction protocol based on the employment of the meso-fluidic system is under investigation in the author's group. In addition, 10 μg ml$^{-1}$ bovine serum albumin, 20 mM EDTA, 0.2 μg ml$^{-1}$ SDS, 2 μg ml$^{-1}$ CTAB and 0.1% Triton X-100

Table 1
Characteristic performance data of the meso-fluidic system for DNA assay

| | |
|---|---|
| Linear calibration range (μg ml$^{-1}$) | 0.2–6.0 |
| Regression equation | $0.07763C_{DNA}$–0.00653 |
| Correlation coefficient | 0.9990 |
| Sampling frequency (h$^{-1}$) | 30 |
| DNA consumption (μl) | 5 (5 μg ml$^{-1}$) |
| Crystal violet consumption (μl) | 10 ($5 \times 10^{-5}$ mol l$^{-1}$) |
| R.S.D. (2.0 μg ml$^{-1}$, $n = 11$) (%) | 1.2 |
| Detection limit (3σ, $n = 11$) (μg ml$^{-1}$) | 0.07 |
| Detection limit of the only on-line procedure ([16]) (μg ml$^{-1}$) | 2.6 |

Table 2
Determination of λ-DNA/*Hin*dIII in synthetic samples

| Sample | DNA content ($\mu g\,ml^{-1}$) | This procedure | | | Documented procedure [1] | |
|---|---|---|---|---|---|---|
| | | Found ($\mu g\,ml^{-1}$) | Recovery (%) | R.S.D.[d] (%) | Found ($\mu g\,ml^{-1}$) | Recovery (%) |
| 1[a] | 3.0 | 2.96 | 98.7 | 1.1 | 2.82 | 94.3 |
| 2[b] | 3.0 | 2.90 | 96.7 | 0.8 | 2.93 | 97.7 |
| 3[c] | 3.0 | 3.05 | 101.7 | 1.3 | 3.14 | 104.7 |

[a] Spiked interfernences: BSA, 2.0 $\mu g\,ml^{-1}$; $Ca^{2+}$, 1.8 $\mu mol\,l^{-1}$; $Mg^{2+}$, 1.0 $\mu mol\,l^{-1}$.
[b] Spiked interfernences: EDTA,10 $mmol\,l^{-1}$; $Ca^{2+}$,1.8 $\mu mol\,l^{-1}$; $Mg^{2+}$, 1.0 $\mu mol\,l^{-1}$.
[c] Spiked interfernences: CTAB, 2.0 $\mu g\,ml^{-1}$; SDS, 0.2 $\mu g\,ml^{-1}$; Triton X-100, 0.1%.
[d] $n = 5$.

do not interfere with the determination of 3.0 $\mu g\,ml^{-1}$ λ-DNA/*Hin*dIII.

### 3.3. The performance of the proposed procedure

The recorded spectra under the aforementioned optimal experimental conditions were illustrated in Fig. 7, and the performance data of the present approach for DNA assay by employing a lab-on-valve meso-fluidic system were summarized in Table 1.

It is obvious that the meso-fluidic system is characterized by minimized fluid consumption, i.e., only a very few microliters of sample volume is required, thus, give rise to a considerable sample economy in comparsion with flow injection or even the conventional sequential injection system, not to say the batch mode procedure based on the same decoloration reaction system which consumed 200 times more DNA sample [4]. This provides a promising avenue for bioassay, especially for routine analysis with very limited amount of sample volume. In the mean time, the employment of a LOV unit also facilitated in situ real time monitoring of the reaction process. Futhermore, it entails a much higher sensitivity as compared to the only flow injection procedure reported so far based on the decoloration of a similar dye, methyl violet, the detection limit of which is 40 times higher than the present procedure

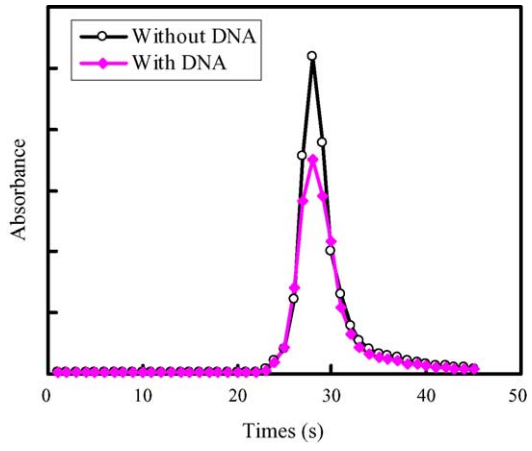


Fig. 7. The recorded spectra under the optimal experimental conditions. Concentration and volume of crystal violet: $5 \times 10^{-5}$ $mol\,l^{-1}$, 10 μl; concentration and volume of λ-DNA/*Hin*dIII: 5 $\mu g\,ml^{-1}$, 5 μl; pH: 8.6; sample loading flow rate: 7 $\mu l\,s^{-1}$.

[16]. The reason might be attributed to the employment of a 350 cm reaction coil to transport a 60 μl DNA sample zone into the detector, during which process the dispersion resulted in a significant decrease of sensitivity.

The applicability of the procedure was demonstrated and validated by the analyses of synthetic samples, which were prepared by spiking appropriate amounts of foreign ions and/or substances in a solution containing 3.0 $\mu g\,ml^{-1}$ λ-DNA/*Hin*dIII. The samples were also analyzed in parallel by using a documented spectrophotometric procedure [1] and agreements were achieved between the two procedures. The results obtained were summarized in Table 2.

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## References

[1] A.A. Killeen, Microchem. J. 52 (1995) 333.
[2] Y.-M. Hao, H.-X. Shen, Anal. Chim. Acta 422 (2000) 159.
[3] M. Samuel, M.-Q. Lu, C.J. Pachuk, C. Satishchandran, Anal. Biochem. 313 (2003) 301.
[4] G.-R. Fang, G.-W. Song, Y. Li, Fenxi Ceshi Xuebao 19 (2000) 45.
[5] J.-H. Yang, C.-G. Lin, G.-L. Zhang, Z.-Q. Gao, X. Wu, X.-R. Huang, Spectrochim. Acta Part A 54 (1998) 2019.
[6] E.S. Morozkin, P.P. Laktionov, E.Y. Rykova, V.V. Vlassov, Anal. Biochem. 322 (2003) 48.
[7] Q.-E. Cao, Y.-K. Zhao, Y.-Y. Xu, C.-Z. Li, Z.-D. Hu, Q.-H. Xu, Anal. Biochem. 277 (2000) 214.
[8] F. Vitzthum, G. Geiger, H. Bisswanger, H. Brunner, J. Bernhagen, Anal. Biochem. 276 (1999) 59.
[9] Y.-J. Ma, M. Zhou, X.-Y. Jin, Z.-Y. Zhang, X.-L. Teng, H. Chen, Anal. Chim. Acta 501 (2004) 25.
[10] H. Chen, M. Zhou, X.-Y. Jin, Y.-M. Song, Z.-Y. Zhang, Y.-J. Ma, Anal. Chim. Acta 478 (2003) 31.
[11] C.-Z. Huang, K.-A. Li, S.-Y. Tong, Anal. Chem. 68 (1996) 2259.
[12] X.-F. Long, Q. Miao, S.-P. Bi, D.-S. Li, C.-H. Zhang, H. Zhao, Talanta 64 (2004) 366.
[13] X. Chu, G.-L. Shen, J.-H. Jiang, R.-Q. Yu, Anal. Lett. 32 (1999) 717.
[14] N.A. El-Maali, J. Wang, Sens. Actuator B. 76 (2001) 211.

[15] R.-H. Yang, K.-M. Wang, D. Xiao, K. Luo, X.-H. Yang, Anal. Chim. Acta 432 (2001) 135.
[16] X.-P. Zhu, J.-W. Wang, M.-G. Fu, J.-G. Xue, Z.-X. Wang, Nanchang Daxue Xuebao (Sci. ed.) 25 (2001) 277.
[17] C.-H. Wu, J. Ruzicka, Analyst 126 (20011947).
[18] C.M. Schulz, J. Ruzicka, Analyst 127 (2002) 1293.
[19] A.D. Carroll, L. Scampavia, J. Ruzicka, Analyst 127 (2002) 1228.
[20] J. Ruzicka, Analyst 125 (2000) 1053.
[21] J.-H. Wang, Z.-L. Fang, Chin. J. Anal. Chem. 32 (2004) 1401.
[22] J.-H. Wang, Anal. Bioanal. Chem., in press.
[23] J.A. Bergeron, M.J. Singer, Biophys. Biochem. Cytol. 4 (1958) 433.