Biosensors and Bioelectronics 33 (2012) 274–278

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Short communication

Detection of single-nucleotide polymorphisms with novel leaky surface acoustic wave biosensors, DNA ligation and enzymatic signal amplification

Qinghua Xu[a,1], Kai Chang[a,1], Weiping Lu[a,1], Wei Chen[a], Yi Ding[b], Shuangrong Jia[a], Kejun Zhang[a], Fake Li[a], Jianfeng Shi[b], Liang Cao[b], Shaoli Deng[a,*], Ming Chen[a,*]

[a] Department of Clinical Laboratory Medicine, Institute of Surgery Research, Daping Hospital, The Third Military Medical University, Chongqing 400042, China
[b] The 26th Research Institute, Chinese Electronic Scientific and Technical Group Company, Chongqing, 400060, China

ARTICLE INFO

Article history:
Received 9 August 2011
Received in revised form
19 December 2011
Accepted 20 December 2011
Available online 27 December 2011

Keywords:
Single nucleotide polymorphism
Leak surface acoustic wave biosensor
DNA ligation

ABSTRACT

This manuscript describes a new technique for detecting single-nucleotide polymorphisms (SNPs) by integrating a leaky surface acoustic wave (LSAW) biosensor, enzymatic DNA ligation and enzymatic signal amplification. In this technique, the DNA target is hybridized with a capture probe immobilized on the surface of a LSAW biosensor. Then, the hybridized sequence is ligated to biotinylated allele-specific detection probe using Taq DNA ligase. The ligation does not take place if there is a single-nucleotide mismatch between the target and the capture probe. The ligated detection probe is transformed into a streptavidin–horseradish peroxidase (SA–HRP) terminal group via a biotin–streptavidin complex. Then, the SA–HRP group catalyzes the polymerization of 3,3-diaminobenzidine (DAB) to form a surface precipitate, thus effectively increasing the sensitivity of detecting surface mass changes and allowing detection of SNPs. Optimal detection conditions were found to be: 0.3 mol/L sodium ion concentration in PBS, pH 7.6, capture probe concentration 0.5 μmol/L and target sequence concentration 1.0 μmol/L. The detection limit was found to be 1×10^{-12} mol/L. Using this technique, we were able to detect a single-point mutation at nucleotide A2293G in Japanese encephalitis virus.

1. Introduction

Single-nucleotide polymorphisms (SNPs) are considered to be third-generation genetic markers (Gao et al., 2005). SNPs have been exploited in various gene analysis applications, such as the identification of pathogenic microorganisms, analyses of drug resistance in clinics and early diagnoses of genetic disorders (Hirschhorn and Daly, 2005; Shastry, 2009). Therefore, a fast, simple and economic technique for identifying SNPs is required for accurate clinical diagnoses of genetic disorders and the design of appropriate treatments.

A variety of biosensors have been widely used in the detection of SNPs because of their high selectivity and sensitivity, short analysis cycle, low cost and suitability for continuous analyses of complex systems. Currently, biosensing of SNPs is based on the following principles: single-base extension (Patolsky et al., 2001), molecular beacon (Mao et al., 2008), protein-DNA binding (Su et al., 2004) and rolling circle amplification (Li et al., 2010; Zhang et al., 2009). Although these techniques are able to detect single-nucleotide mutations, they have several major disadvantages, such as complex procedures, long analysis cycle and insufficient sensitivity/specificity. Compared with these techniques, SNP detection via enzymatic DNA ligation is simple to operate and offers high specificity. When a capture and detection probe perfectly match a target sequence, they can be connected via a phosphate diester structure by DNA ligase. In contrast, if the probe hybridizes with the target sequence with a single-nucleotide mismatch, the two probes cannot be connected. This provides a reliable and highly specific approach of detecting single-nucleotide mutations (Toubanaki et al., 2009; Zhang et al., 2008; Zhou et al., 2009).

Leaky surface acoustic wave (LSAW) biosensors are a relatively new technology (Inoue et al., 2007; Kushibiki and Ohashi, 2006). The LSAW biosensors excite a LSAW based on a special orientation of a piezoelectric crystal (e.g. a 36° rotated Y-cut X propagation lithium tantalate ($LiTaO_3$) piezoelectric single crystal). A LSAW is a minimal vibration component that is perpendicular to the surface. Therefore, the wave is potentially sensitive to any change in the surface, such as mass loading, viscosity and conductivity changes. With even a small mass loading, the LSAW experiences changes in boundary conditions and thus responds to shifts in phase angle. These variations offer a mechanism of sensitive detection of biological molecules based on their specific interaction on the surface (Chung et al., 2006; Makkonen et al., 2006; Taziev, 2007). Compared with traditional quartz crystal microbalances (Chen et al., 2005; Luo et al., 2006; Yao et al., 2008), LSAW biosensors have

* Corresponding authors. Tel.: +86 23 68757601; fax: +86 23 68716530.
E-mail addresses: dengsl072@yahoo.com.cn (S. Deng), chenming1971@yahoo.com (M. Chen).
[1] Qinghua Xu, Kai Chang and Weiping Lu contributed equally to this work.

0956-5663/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2011.12.036

remarkably high base frequencies, between 100 MHz and 2 GHz, and can achieve substantially higher sensitivities.

In a previous study, we developed a peptide nucleic acid LSAW biosensor for the rapid detection of human papilloma virus (Wang et al., 2009). In this study, our objective was to develop a simple and sensitive technique exhibiting high specificity for the rapid analyses of Japanese encephalitis virus (JEV) in clinical laboratories. To fulfill this goal, we developed a new SNPs biosensing technique by combining enzymatic DNA ligation and biological signal amplification.

2. Materials and methods

2.1. Materials

Attenuated live-vaccine strain (SA14-14-2, serial No: AF315119) of JEV and virulent strain of JEV (P3, serial No: AY243844) were obtained from the Center of Disease Control and Prevention (Chongqing, China). The detection system was described in our previous study (Wang et al., 2009).

2.2. Optimization of detection conditions

2.2.1. Effect of pH value of PBS buffer

The detection channel of LSAW biosensor was surface-immobilized with capture probe (5′-SH-(CH2)6-AAACACTTGGTGAACGGCTC-3′) (0.5 uM) via thiol groups; PBS buffer was added to the reference channel. The sensor was rinsed three times with deionized water and dried in a nitrogen stream. Then, both channels were filled with 72.0 μL of PBS (10 mM) with a pH range (6.4, 6.8, 7.2, 7.6, 8.0 and 8.4) and the oscillating signals from both channels were monitored. After the phase shifts were stabilized, 1.0 μmol/L Target-G (strain SA14-14-2: 5′-GGGGTCTTCAACTCCATAGG AA**G**AGCCGTTCACCAAGTGTTT-3′) was added to the channels. The sensor was maintained at 37 °C to initiate hybridization, the phase shifts of both the detection channel (P_1) and reference channel (P_2) were determined, and the relative phase shift attributed to the hybridization between capture probe and Target-G was calculated by: $\Delta\text{Phase} = P_1 - P_2$. The time required for the reaction to reach equilibrium was also recorded. The experiment was repeated four times.

2.2.2. Effect of ionic concentration of PBS buffer

The procedures are similar to those mentioned above. For immobilization of the capture probe, both channels were filled with 72.0 μL of PBS (pH 7.6) containing different concentrations of sodium chloride (0.1, 0.2, 0.3, 0.6 mol/L). After the phase shift was stabilized, 1.0 μmol/L Target-G was added into both channels. The ΔPhase and equilibrium time were recorded.

2.2.3. Effect of probe concentration

Capture probe, with a final concentration of 0.1, 0.5, 1.0 and 1.5 μmol/L, was immobilized on the surface of the detection channels. PBS (10 mM, pH 7.6) was added to the corresponding reference channel. After the phase shift was stabilized, 1.0 μmol/L Target-G was added into both channels. Similarly, the ΔPhase and equilibrium time were recorded.

2.2.4. Effect of target sequence concentration

Capture probe (0.5 μM) was first surface-immobilized on the detection channels of an LSAW biosensor as described above. Then, 0.1, 0.5, 1.0, 1.5 and 2.0 μmol/L of Target-G were added to both channels. The ΔPhase and equilibrium time were recorded.

2.3. SNPs analyses with LSAW biosensors

Fig. S1 (in Supplementary materials) showed the principles of SNPs detection with LSAW biosensor. The immobilization of capture probe was described above. 1.0 μmol/L Target-G and Target-A (strain P3: 5′-GGGGTCTTCAACTCCATAGGAA**A**A GCCGTTCACCAAGTGTTT-3′) were added separately to the detection channel and reference channel. Then, 0.5 μmol/L detection probe (5′-Phosphate-TTCCTATG GAGTTGAAGACCCC-biotin-3′) was added to both channels. After rinsing with PBS, both channels were filled with a solution containing 800.0 U/mL Taq DNA ligase, and the sensor was maintained at 45 °C for 35 min for ligation. Both channels were treated with 0.05 mol/L NaOH to initiate denaturation, and then filled with 60.0 μL of SA–HRP solution. The sensor was maintained at 37 °C for 30 min. After rinsing again with PBS buffer, both channels were filled with 20.0 μL of DAB solution. The ΔPhase and equilibrium time were recorded.

2.4. Determination of detection limit of LSAW biosensors

Different concentrations (1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9}, 1×10^{-10}, 1×10^{-11} and 1×10^{-12} mol/L) of Target-G were analyzed with the LSAW sensors as described above to evaluate the detection limit for this target sequence.

2.5. Detection of SNPs in JEV strains

A mutant strain (SA14-14-2) and a wild-type strain (P3) of JEV were analyzed with our LSAW biosensors to investigate detection potential with clinically relevant viruses. RNA was extracted from each strain following instruction of the QIAGEN kits and amplified by RT-PCR. The PCR products were denatured at 95 °C for 5 min, and then flash-frozen to yield single DNA chains of SA14-14-2 and P3. The corresponding single chains were analyzed with the LSAW sensors as described above.

2.6. Data analyses

Phase shift data were presented as mean ± standard deviation and analyzed by one-way ANOVA (SPSS 13.0, SPSS, Chicago, IL, USA). A p-value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Effect of PBS buffer pH

Our investigation of the optimal conditions for the hybridization between nucleic acid molecules revealed that the suitable pH range value for hybridization was narrow. The ΔPhase (Fig. 1Aa) increased significantly from pH 6.4 to 7.6 ($p < 0.01$) and decreased at pH > 7.6. The time required to reach equilibrium increased slowly from pH 6.4 to 8.4 (Fig. 1Ab). The coefficients of variation (CVs) were shown in Table S1(Supplementary materials). Considering these two parameters, we determined the optimal pH to be 7.6. This indicates that the most suitable environment for hybridization occurs in slightly alkaline conditions, probably because under such conditions the analytes or the probes reach their isoelectric point(s) and can hybridize more efficiently. Moreover, change in surface charge density with pH may be also partially responsible for this pH-dependent behavior.

3.2. Effect of PBS buffer ionic concentration

Sodium ion concentration substantially affected the hybridization process. The ΔPhase increased significantly ($p < 0.01$) (Fig. 1Ba)

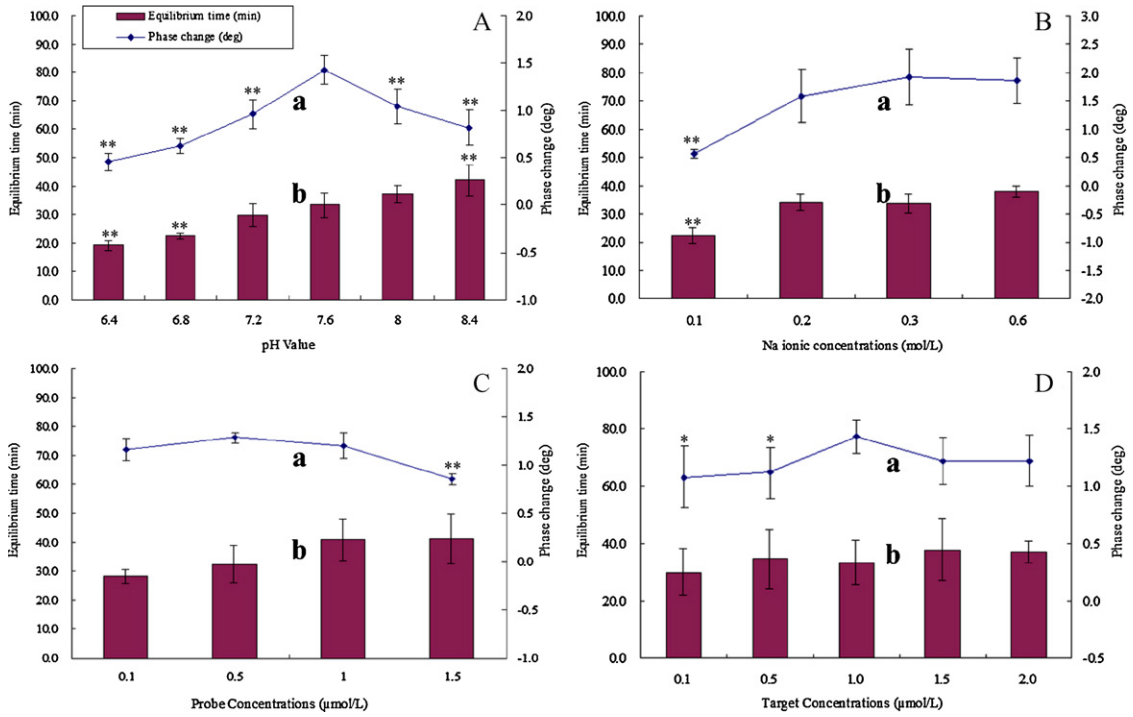

Fig. 1. Optimization of detection conditions; A: Effect of different pH value on (a) ΔPhase and (b) equilibrium time vs. pH 7.6 group; B: Effect of different ion concentrations on (a) ΔPhase and (b) equilibrium time vs. sodium concentration of 0.3 mol/L group; C: Effect of different capture probe concentrations on (a) ΔPhase and (b) equilibrium time vs. 0.5 μmol/L probe group; D: Effect of different target concentrations on (a) ΔPhase and (b) equilibrium time vs. 1.0 μmol/L Target-G group. Error bars indicate the standard deviation ($n = 4$), $^{*}p < 0.05$. $^{**}p < 0.01$.

when sodium ion concentration increased from 0.1 mol/L to 0.3 mol/L and reached the plateau at 0.3 mol/L. The time to equilibrium was significantly shorter at a sodium ion concentration of 0.1 mol/L ($p < 0.01$) (Fig. 1Bb) and remained similar at other concentrations. The CVs were shown in Table S1. The results showed that 0.3 mol/L was optimal ionic strength for probe-target hybridization, probably because of the electrostatic interactions between the DNA fragments. DNA fragments contain many negatively charged phosphate molecules that repel each other in solution. At an appropriate ionic strength, cations in the solution can be electrostatically attracted to phosphate groups, thus reducing the repulsion between fragments and facilitate their hybridization.

3.3. Effect of probe concentration

The phase shift increased slightly (Fig. 1Ca) with capture probe concentrations ranging from 0.1 μmol/L to 0.5 μmol/L and then decreased from 0.5 μmol/L to 1.5 μmol/L. Compared with the lowest concentration of 0.1 μmol/L, the ΔPhase at 1.5 μmol/L was significantly lower ($p < 0.01$). The time to equilibrium (Fig. 1Cb) increased slightly from 0.1 μmol/L to 1.5 μmol/L, but the trend was not statistically significant ($p > 0.05$). The CVs were shown in Table S1. The results showed that 0.5 μmol/L probe produced the highest sensitivity, whilst a further increase in probe concentration yielded a reduced sensitivity. This spatial arrangement of the probe molecules may explain the probe-concentration-dependent behavior. When the probe concentration rises above the threshold, the probe molecules are densely packed on the gold surface and provide limited space for the target sequences to enter and hybridize. In addition to this spatial restriction, the electrostatic repulsion between DNA fragments may also contribute to the changes in sensitivity with probe concentration.

3.4. Effect of target sequence concentration

Target sequence concentration plays an important role in the hybridization of nucleic acid molecules. The ΔPhase increased significantly ($p < 0.05$) (Fig. 1Da) when Target-G concentrations increased from 0.1 μmol/L to 1.0 μmol/L, decreased slightly between 1.0 μmol/L and 1.5 μmol/L, and remained stable between 1.5 μmol/L and 2.0 μmol/L (Fig. 1Da). The time to equilibrium was similar at all concentrations (Fig. 1Db). The optimal Target-G concentration was determined to be 1.0 μmol/L.

3.5. Detection of SNPs in experimental target sequences

The phase shifts were similar after hybridization with Target-A ($2.00^{\circ} \pm 0.24^{\circ}$) or Target-G ($2.20^{\circ} \pm 0.16^{\circ}$). However, after further enzymatic amplification, the phase shifts became significantly different; $0.70^{\circ} \pm 0.08^{\circ}$ in the case of Target-A compared with $15.00^{\circ} \pm 1.47^{\circ}$ in the case of Target-G (Table 1), These results confirm that the LSAW biosensor could reliably detect SNPs in DNA fragments. The CVs of Target-A and Target-G were 12.00% and 7.27% for hybridization, 11.43% and 9.80% for enzymatic amplification, respectively.

Sensitivity is an important characteristic of biosensors. Although the LSAW biosensor can provide high sensitivities, it is still difficult to detect single-nucleotide mutations without further amplification. Effective signal amplification is critical for enhancing the sensitivities of SNP detection. Previous studies have addressed this problem by tagging probes with markers, such as hydrogen peroxidase (Zhang et al., 2008), liposomes (Kumanan et al., 2009; Nugen et al., 2007), biotin or nanoparticles (Li et al., 2006; Yeh et al., 2010). Some have also amplified the signal by using peptide nucleic acids (Al Attar et al., 2008; Kerman et al., 2008). In this study, HRP-catalyzed DAB polymerization-precipitation was used for signal amplification. The LSAW biosensor showed an excellent response at $>1 \times 10^{-12}$ mol/L Target-G, and the signal could

Table 1
Signals obtained from different target sequences before and after enzymatic amplification (mean ± standard deviation).

	Target-A						Target-G					
	Hybridization			Enzyme amplification			Hybridization			Enzyme amplification		
	Mean	S.D.	CV (%)	Mean	S.D.	CV (%)	Mean	S.D.	CV (%)	Mean	S.D.	CV (%)
ΔPhase (degree)	2.00	0.24	12.00	0.70	0.08	11.43	2.20	0.16	7.27	15.00*	1.47	9.80
Equilibrium time (min)	33.00	6.78	20.55	40.00	4.08	10.20	33.25	3.10	9.32	50.00	10.80	21.60

Measurements are ΔPhase and equilibrium time of the LSAW biosensor after hybridization between detection probe and different target sequences and subsequent ligation.
* Indicates $p < 0.01$ compared with hybridization with Target-A after enzymatic amplification.

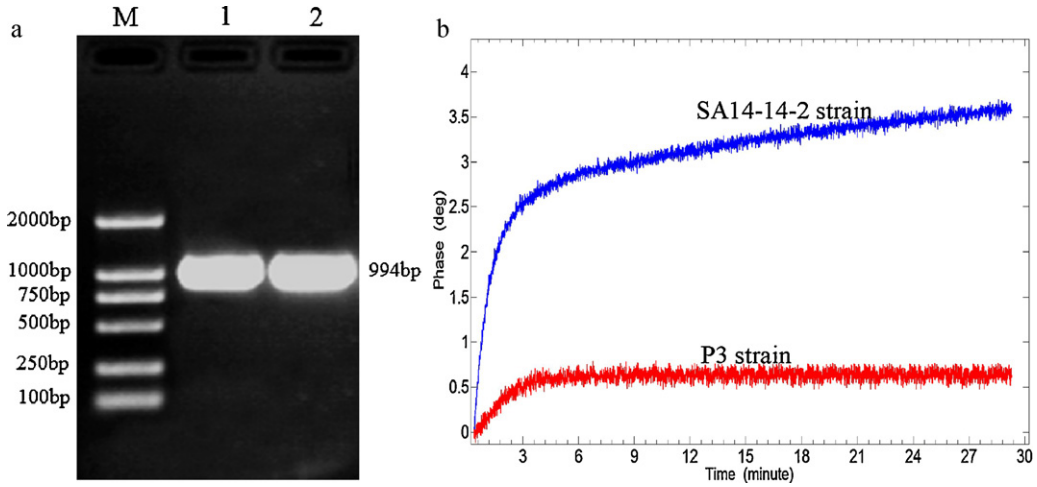

Fig. 2. (a) Electrophoresis gel of RNAs extracted from (lane 1) JEV strain SA14-14-2 and (lane 2) strain P3; M shows DNA Markers (DL 2000); (b) real-time detection signals for strains SA14-14-2 and P3 of Japanese encephalitis virus (1 nmol/L).

not be differentiated from the background below this Target-G concentration. Thus, the detection limit for Target-G was determined to be 1×10^{-12} mol/L. Additionally, the phase shift was linear with the logarithm of the Target-G concentration within the range of 1×10^{-12} to 1×10^{-6} mol/L ($\Delta P = 1.853 \lg C + 22.235$, $r = 0.976$) (shown in Fig. S2 in Supplementary materials).

3.6. Analyses of clinically relevant samples

The virulence-associated genes of JEV are concentrated in the E-protein coding region (Roehrig, 2003; Zhao et al., 2005), and a single-point mutation in this region can result in substantial changes in neurovirulence and invasiveness. A mutation at nucleotide A2293G causes an amino acid change from Lys to Arg at the position E439 in E protein. So far, this single-point mutation is found in all known attenuated JEV strains (Chambers et al., 2007). Therefore, it provides a critical marker for the detection of SNPs in different strains of JEV.

In this study, we demonstrated the detection of the viral strains SA14-14-2 and P3 using LSAW biosensors. The results revealed that analysis of the PCR product (Fig. 2a) of strain SA14-14-2 with a LSAW sensor (Fig. 2b) generated a phase shift of $3.65^\circ \pm 0.13^\circ$ ($n = 4$), compared with $0.56^\circ \pm 0.08^\circ$ ($n = 4$) from strain P3. The difference between the two results was statistically significant ($p < 0.01$). JEV has been detected with immunosensors in previous studies, but immunosensors could not detect the single nucleotide mutation (Li et al., 2011; Yuan et al., 2005). However, compared with the detection of the Target-G during the optimization of experimental conditions, the detection of SA14-14-2 produced a substantially smaller signal. The reduced signal intensity may be attributed to two factors. Firstly, Target-G is a shorter fragment (42 bp) and thus can more easily hybridize with capture probe. In comparison, the target fragment for SA14-14-2 is longer (994 bp) and hybridizes with the capture probe at a lower efficiency. Secondly, single-chain PCR products may undergo a gradual renaturation and compete with capture probe for hybridization, and thus negatively affect the detection sensitivity.

4. Conclusions

In this study, we developed a new technique based on a LSAW biosensor, enzymatic DNA ligation and enzymatic signal amplification for the detection of SNPs. This technique is easy to operate, requires no labels and is suitable for real-time dynamic detection. However, the CV values of hybridization and enzyme amplification were not very good which means the reproducibility of our detection system still leaves room for improvement. Furthermore, a PCR procedure is still required, which means a long detection period compared to other experiments (Chen et al., 2005). This new technique can be potentially used for diagnosis of other pathogenic microorganisms or some genetic diseases such as the thalassemia.

Acknowledgements

This study was supported by the National High Technology Research and Development Project (863, grant number: 2007AA02Z416), National Natural Sciences Foundation of China (grant numbers: 81071428, 30400107), Eleventh Five-year Plan of Medical Research PLA (grant number: 06G073), and Key Scientific and Technological Project of Chongqing (grant number: CSTC, 2009BA5007, CSTC, 2011AC5033).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.12.036.

References

Al Attar, H.A., Norden, J., O'Brien, S., Monkman, A.P., 2008. Biosens. Bioelectron. 23 (10), 1466–1472.

Chambers, T.J., Droll, D.A., Jiang, X., Wold, W.S., Nickells, J.A., 2007. Virology 366 (1), 51–61.

Chen, M., Liu, M., Yu, L., Cai, G., Chen, Q., Wu, R., Wang, F., Zhang, B., Jiang, T., Fu, W., 2005. J. Nanosci. Nanotechnol. 5 (8), 1266–1272.

Chung, C.J., Kao, K.S., Cheng, C.C., Chen, Y.C., 2006. IEEE Trans. Ultrason. Ferroelectr. Freq. Control 53 (2), 502–505.

Gao, X.L., Jing, F.X., Yang, J.B., Zhao, J.L., 2005. Yi Chuan 27 (1), 110–122.

Hirschhorn, J.N., Daly, M.J., 2005. Nat. Rev. Genet. 6 (2), 95–108.

Inoue, S., Tsutsumi, J., Matsuda, T., Ueda, M., Ikata, O., Satoh, Y., 2007. IEEE Trans. Ultrason. Ferroelectr. Freq. Control 54 (8), 1692–1699.

Kerman, K., Saito, M., Tamiya, E., 2008. Anal. Bioanal. Chem. 391 (8), 2759–2767.

Kumanan, V., Nugen, S.R., Baeumner, A.J., Chang, Y.F., 2009. J. Vet. Sci. 10 (1), 35–42.

Kushibiki, J., Ohashi, Y., 2006. IEEE Trans. Ultrason. Ferroelectr. Freq. Control 53 (2), 385–392.

Li, Y., Wark, A.W., Lee, H.J., Corn, R.M., 2006. Anal. Chem. 78 (9), 3158–3164.

Li, J., Deng, T., Chu, X., Yang, R., Jiang, J., Shen, G., Yu, R., 2010. Anal. Chem. 82 (7), 2811–2816.

Li, F., Mei, L., Li, Y., Zhao, K., Chen, H., Wu, P., Hu, Y., Cao, S., 2011. Biosens. Bioelectron. 26 (10), 4253–4256.

Luo, Y., Chen, M., Wen, Q., Zhao, M., Zhang, B., Li, X., Wang, F., Huang, Q., Yao, C., Jiang, T., Cai, G., Fu, W., 2006. Clin. Chem. 52 (12), 2273–2280.

Makkonen, T., Plessky, V.P., Steichen, W., Grigorievski, V.I., Solal, M., Salomaa, M.M., 2006. IEEE Trans. Ultrason. Ferroelectr. Freq. Control 53 (2), 393–401.

Mao, X., Jiang, J., Xu, X., Chu, X., Luo, Y., Shen, G., Yu, R., 2008. Biosens. Bioelectron. 23 (10), 1555–1561.

Nugen, S.R., Leonard, B., Baeumner, A.J., 2007. Biosens. Bioelectron. 22 (11), 2442–2448.

Patolsky, F., Lichtenstein, A., Willner, I., 2001. Nat. Biotechnol. 19 (3), 253–257.

Roehrig, J.T., 2003. Adv. Virus Res. 59, 141–175.

Shastry, B.S., 2009. Methods Mol. Biol. 578, 3–22.

Su, X., Robelek, R., Wu, Y., Wang, G., Knoll, W., 2004. Anal. Chem. 76 (2), 489–494.

Taziev, R.M., 2007. IEEE Trans. Ultrason. Ferroelectr. Freq. Control 54 (10), 2060–2069.

Toubanaki, D.K., Christopoulos, T.K., Ioannou, P.C., Flordellis, C.S., 2009. Anal. Chem. 81 (1), 218–224.

Wang, Y., Chen, M., Zhang, L., Ding, Y., Luo, Y., Xu, Q., Shi, J., Cao, L., Fu, W., 2009. Biosens. Bioelectron. 24 (12), 3455–3460.

Yao, C., Zhu, T., Tang, J., Wu, R., Chen, Q., Chen, M., Zhang, B., Huang, J., Fu, W., 2008. Biosens. Bioelectron. 23 (6), 879–885.

Yeh, C.H., Chang, Y.H., Chang, T.C., Lin, H.P., Lin, Y.C., 2010. Analyst 135 (10), 2717–2722.

Yuan, R., Zhang, L., Li, Q., Chai, Y., Cao, S., 2005. Anal. Chim. Acta 531 (1), 1–5.

Zhang, P., Chu, X., Xu, X., Shen, G., Yu, R., 2008. Biosens. Bioelectron. 23 (10), 1435–1441.

Zhang, S., Wu, Z., Shen, G., Yu, R., 2009. Biosens. Bioelectron. 24 (11), 3201–3207.

Zhao, Z., Date, T., Li, Y., Kato, T., Miyamoto, M., Yasui, K., Wakita, T., 2005. J. Gen. Virol. 86 (Pt 8), 2209–2220.

Zhou, H., Xing, D., Zhu, D., Zhou, X., 2009. Talanta 78 (4–5), 1253–1258.