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Establishment of ELISA on 384-Well Microplate for AFP, CEA, CA 19-9, CA 15-3, CA 125, and PSA-ACT: Higher Sensitivity and Lower Reagent Cost

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The 384-well microplate contains four times as many wells as the regular 96-well microplate. Establishing enzyme linked immunosorbent assay (ELISA) on 384-well microplate should lead to savings in reagents and specimens. To determine that ELISAs on 384-well microplate have acceptable assay precision, ELISAs for tumor markers, including AFP, PSA-ACT, CEA, CA 125, CA 15-3, and CA 19-9, were compared to the same ELISAs established on 96-well microplate. We found that ELISAs established on 384-well microplate had similar sensitivity and covered similar concentration ranges as ELISAs on 96-well microplate. All within-day and day-to-day precisions for the 384-ELISA had %CV less than 10%. Compared to ELISA on 96-well microplate, 384-ELISA used less reagents, less specimen, and exhibited approximately a two-fold increase in sensitivity. Overall cost of the 384-384-ELISA was also greatly reduced. Our results suggest that 384-ELISA is suitable for use in routine clinical laboratories. J. Clin. Lab. Anal. 17:241–246, 2003. © 2003 Wiley-Liss, Inc.

Key words: tumor marker; ELISA; 384-well microplate; 96-well microplate

INTRODUCTION

Enzyme linked immunosorbent assay (ELISA) has been successfully established on 96-well microplate in the past. Recently, microplates containing 384 wells and 1536 wells also became available. There is temptation, consequently, to use these microplates for establishing ELISAs in order to save expensive reagents and precious specimens. In addition, improved washing apparatus are now available for 384-well microplate.

There is a need for clinical laboratories in under developed countries to establish ELISA on microplate. Most of these countries, however, cannot afford automated instruments and reagent agreement. Even if they do have the instruments, there is the additional problem of lack of regular and emergency service for the instruments.

All equipment, either manually operated or automated (workstation) for coating, liquid handling, washing, and final signal reading are now commercially available. They can be used for all assays established on microplate without being limited to specific groups of commercially designed analytes. High quality 384 microplate can be purchased from numerous commercial sources (e.g., NUNC™ [Naperville, IL]; Whatman Inc. [Clifton, NJ]; Corning [Corning, NY]; Packard Instrument Co. [Meriden, CT]; and Thermo Labsystems [Franklin, MA]). All major companies (e.g., Tecan [Research Triangle Park, NC]; Zarmark Corp. [Hopiniton, MA]; Molecular Device Corp. [Sunnyvale, CA]; Packard Instrument Co.; Perkin Elmer Life Sciences [Norwalk, CT]; Bio-Rad Laboratories Inc. [Richmond, CA]; BioSystems [Sunnyvale, CA]; and Thermo Labsystems, BIO-TEK [Winooski, VT]) produce an entire fleet of apparatus, from liquid pipetting to spectrophotometric and fluorometric readers. For handling large numbers of specimens, automated 384 coating equipment and automated workstations for 384 ELISA are

Abbreviations: AFP, alpha-fetoprotein; Ab, antibody; CEA, carcinoembrionic antigen; BSA-PBA, 0.01 mol/L phosphate buffer at pH 7.2 containing 1% BSA; ELISA, enzyme linked immunosorbent assay, HRP, horse radish peroxidase; mAb, monoclonal antibody; PSA-ACT, prostate specific antigen-alpha1-antichymotrypsin complex; PBS, phosphate buffered saline

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also available from companies such as Oyster Bay Pump Works, Inc. (Hicksville, NY) and Tecan, respectively.

Although 384-well ELISA has not been adopted in clinical laboratories in the past, their use has been reported in research investigations. For example, Kassack et al. (1) have used 384-well microplate to screen G protein-coupled receptors by measuring intracellular calcium. Garrigues et al. (2) have performed a high-throughout screening of the interaction of drugs with p-glycoproein on 384-well microplate. Since tumor marker measurements is one of the major ELISAs performed in routine clinical laboratories, we wanted to try 384-well microplate for currently used tumor markers.

In this study, in addition to establishing ELISA on 384-well microplate, we also compared the assay precision, the sensitivity, and the concentration range covered of ELISA on 384-well microplate with that on 96-well microplate. We found that ELISA established on 384-well microplate not only had sufficient assay precision for routine clinical use, it also yielded higher sensitivity for ELISA established.

MATERIALS AND METHODS

All antibodies for various ELISA were either from CanAg Diagnostics (Gothenburg, Sweden) or DAKO Corporation (Carpinteria CA). Amdex products were purchased from Amersham International PLC Life Science (Arlington Heights, IL). Purchased from Nalge Nunc International (Naperville, IL) was 384-well microplate, such as Maxisorp (without lid, with optical bottom plate).

All calibrators used in the ELISA for tumor markers were pooled serum (with the exception of the calibrator for CA 125, which was pooled ovarian cystic fluids) from cancer patients calibrated against Abbott kits used in an Abbott Axsym autoanalyzer (Abbott Park, IL).

ELISA Procedure

Except for a difference in volume, all buffer solutions (including sample and antibody dilution, washing, and final enzymatic color development) were the same for both 96- and 384-well microplate ELISA (3): coating buffer—0.1 mol/l carbonate buffer, pH 9.6; washing solution—0.05% v/v Tween 20 in PBS; blocking buffer—1% BSA-PBS, 0.05% casein (w/v) (from stock 2% casein in 0.1 mol/l NaOH) 0.05% Tween 20 (V/V), 5 mmol/l thimerosal, and 2% sucrose (w/v).

Briefly, the ELISA (Fig. 1) was established by first coating the wells with antibody solution (2 μg/mL) at 4°C overnight, followed by the addition of blocking solution (100 μl), and incubated at room temperature for at least 6 hr. Antibody coated microplate was stored covered at 4°C until use. Immediately before sample application, the microplate was washed three times with washing buffer.

For two incubation procedures, sample was added, incubated at 30°C for 30 min, and washed three times before the addition of detection antibody solution. For one incubation procedure, sample and detection antibody were added simultaneously to the well. Washing was also performed after incubating at 30°C for 30 min. Enzyme substrate K-Blue (Pierce, Rockford, IL) was added if the detecting antibody was conjugated with horse radish peroxidase (HRP). After incubation for an appropriate time at room temperature, the reaction was stopped with 1 or 2 mol/l H_2SO_4 and the absorbance read at 450 nm.

It should be pointed out that the optimal dilution of antibodies may be slightly varied then what was indicated in the tables due to the freshness of the antibody solution.

Assay Composition and Condition

Assay compositions and conditions for all ELISA for currently used tumor markers established on 384-well microplate are listed in Tables 1–6.

RESULTS

Calibration Curves on 384-Well Microplate

All calibration curves established for AFP, CEA, CA 125, CA 15-3, CA 19-9, and PSA-ACT on 384-well microplate are shown in Figs. 2–4. Similar concentration

TABLE 1. ELISA for AFP on both 96- and 384-well microplate

	Assay condition	384-well	96-well
Coating Ab	Dako anti-AFP, polyclonal (A008), 2 μg/mL, 4°C, overnight	50 μl/well	100 μl/well
Sample + detecting Ab	Sample + CanAg anti-AFP, mAb (1:40,000), 30°C , 1 hr	10 μl + 40 μl	20 μl + 80 μl
Signal Ab	Amdex anti-mouse HRP conjugate (1:30,000), 30°C, 1 hr	50 μl	100 μl
Color development	K-Blue, r.t., 10 min	50 μl	100 μl
Stop reagent	H_2SO_4	25 μl (2N)	100 μl (1N)

Calibrator: pooled serum. Final absorbance times 0.75.
r.t., room temperature.

TABLE 2. ELISA for CA 19-9 on both 96- and 384-well microplate

	Assay condition	384-well	96-well
Coating Ab	CanAg anti-CA 19-9 (2 µg/mL, 4°C, overnight	50 µl/well	100 µl/well
Sample + detecting Ab	Sample (prediluted 1:3) + anti-CA 19-9, mAb (biotin conjugated), (1:10,000), 30°C, 1 hr	10 µl + 40 µl	20 µl + 80 µl
Signal Ab	Amdex steptavidine-HRP conjugate (1:30,000), 30°C, 1 hr	50 µl	100 µl
Color development	K-Blue, r.t., 10 min	50 µl	100 µl
Stop reagent	H_2SO_4	25 µl (2N)	100 µl (1N)

Ab was diluted with blocking solution. Calibrator was from Centocord kit for CA 19-9 (Malvern, PA). Final absorbance times 0.75.
r.t., room temperature.

TABLE 3. ELISA for CA 15-3 on both 96- and 384-well microplate

	Assay condition	384-well	96-well
Coating Ab	CanAg anti-15-3, 2 µg/mL, 4°C, overnight	50 µl/well	100 µl/well
Sample	Sample + BSA-PBS, 30°C, 1 hr	15 µl + 35 µl	50 µl + 50 µl
Detecting and signal Ab	CanAg anti-CA 15-3-HRP conjugate (1:300), 30°C, 1 hr	50 µl	100 µl
Color development	K-Blue, r.t., 10 min	50 µl	100 µl
Stop reagent	H_2SO_4	25 µl (2N)	100 µl (1N)

Final absorbance time 0.75. Calibrator was from Centocord kit (Malvern, PA) for CA 15-3.
r.t., room temperature.

TABLE 4. ELISA for CA 125 on both 96- and 384-well microplate

	Assay condition	384-well	96-well
Coating Ab	CanAg anti-CA 125, polyclonal, 2 µg/mL, 4°C, overnight	50 µl/well	100 µl/well
Sample	Sample + BSA-PBS, 30°C, 1 hr	15 µl + 35 µl	50 µl + 50 µl
Detecting and signal Ab	CanAg anti-CA 125-HRP conjugated (1:10,000), 30°C, 1 hr	50 µl	100 µl
Color development	K-Blue, r.t., 10 min	50 µl	100 µl
Stop reagent	H_2SO_4	25 µl (2N)	100 µl (1N)

Calibrator is lyophilized cyst fluid, calibrated against Centocord CA 125 kit (Malvern, PA). Final absorbance times 0.75.
r.t., room temperature.

TABLE 5. ELISA for PSA-ACT complex on both 96- and 384-well microplate

	Assay condition	384-well	96-well
Coating Ab	CanAg anti-free PSA, mAb, 2 µg/mL, 4°C, overnight	50 µl/well	100 µl/well
Sample	Sample + BSA-PBS, 30°C, 1 hr	5 µl + 45 µl	20 µl + 80 µl
Detecting Ab	Dako a1-antichymotrypsin, polyclonal, (1:20,000), 30°C, 1 hr	50 µl (1:400)	100 µl
Signal Ab	Dako goat anti-rabbit-HRP (1:6000) 30°C, 1 hr	50 µl	100 µl
Color development	K-Blue, r.t., 10 min	50 µl	100 µl
Stop reagent	H_2SO_4	25 µl (2N)	100 µl (1N)

Both antibodies were diluted with blocking buffer. Final absorbance times 0.75. Calibrator for PSA-ACT complex was isolated from serum of patients with prostate cancer and calibrated against Abbot kit (Abbot Park, IL).
r.t., room temperature.

ranges were covered by both 96- and 384-well ELISAs. The sensitivity of the individual ELISA can be appreciated from the lowest point of calibration curves presented for each ELISA and were all below upper normal cutoff.

384- and 96-Well Calibration Curves Compared

The 384- and 96-well ELISAs of several selected tumor markers were compared. Although both ELISAs were performed under similar conditions, the 384-well

TABLE 6. ELISA for CEA on both 96- and 384-well microplate

	Assay condition	384-well	96-well
Coating Ab	Dako anti-CEA, polyclonal, 2 µg/mL, 4°C, overnight	50 µl/well	100 µl/well
Sample + detecting Ab	Serum + CanAg anti-CEA, mAb (1:2000) 30°C , 1 hr	10 µl + 40 µl	50 µl + 50 µl
Signal Ab	Amdex Anti-mouse HRP conjugate (1:5,000), 30°C, 1 hr	50 µl	100 µl
Color development	K-Blue, r.t., 10 min	50 µl	100 µl
Stop reagent	H_2SO_4	25 µl (2N)	100 µl (1N)

Final absorbance time 0.75. Calibrator for CEA ELISA was from serum of patients with colon cancer and calibrated against Abbot kit (Abbot Park, IL).
r.t., room temperature.

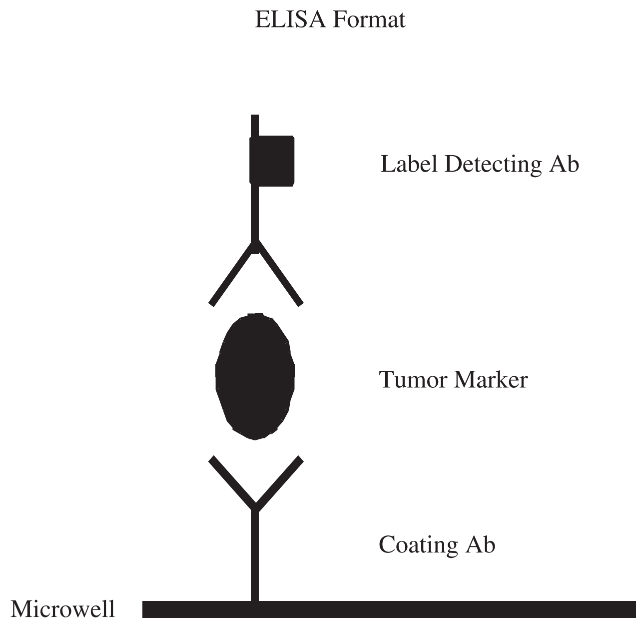

Fig. 1. Test format for both 96- and 384-well ELISAs.

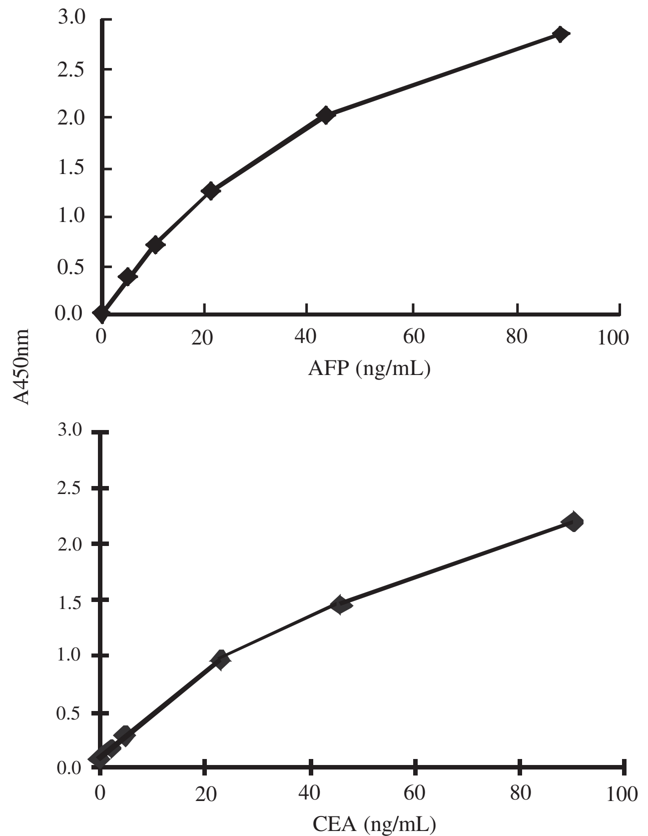

Fig. 2. Calibration curves of 384-well ELISA for CEA and AFP.

ELISAs always had higher sensitivity. As shown in Fig. 5, the calibration curves of 384-well ELISAs exhibited higher absorbance at similar concentrations of the calibrator when compared with the 96-well ELISAs, indicating that 384-ELISA has higher sensitivity.

Precision

We tested the within-day and day-to-day assay precision of 384-ELISA and compared the results with the 96-well ELISA. Both within-day and day-to-day precisions of AFP, CA 125, CA 15-3, and CA 19-9 run on 384-well microplate are listed in Table 7. We did not perform the study for day-to-day precision of CA 19-9 because this tumor marker is known to be less stable over time. Because their precision is well accepted, we also did not perform day-to-day precision for 96-well. The precision with 384-plate also appears acceptable. The CV of 384-ELISA are generally higher than that performed on 96-well microplate for the same tumor marker. However, they all appeared sufficiently low and are generally less than 10%, thus meeting the requirement of an immunoassay for clinical use. It should be noted that precision of all ELISAs was determined manually. When assays are performed by an automated instrument, we expect that the precision of 384-well assays will be improved.

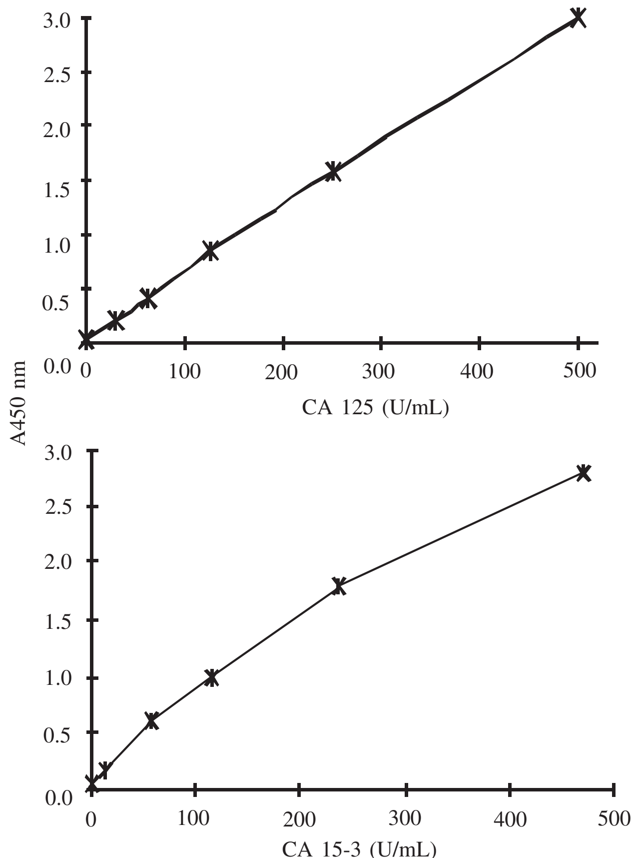

Fig. 3. Calibration curves of 384-well ELISA for CA 125 and CA 15-3.

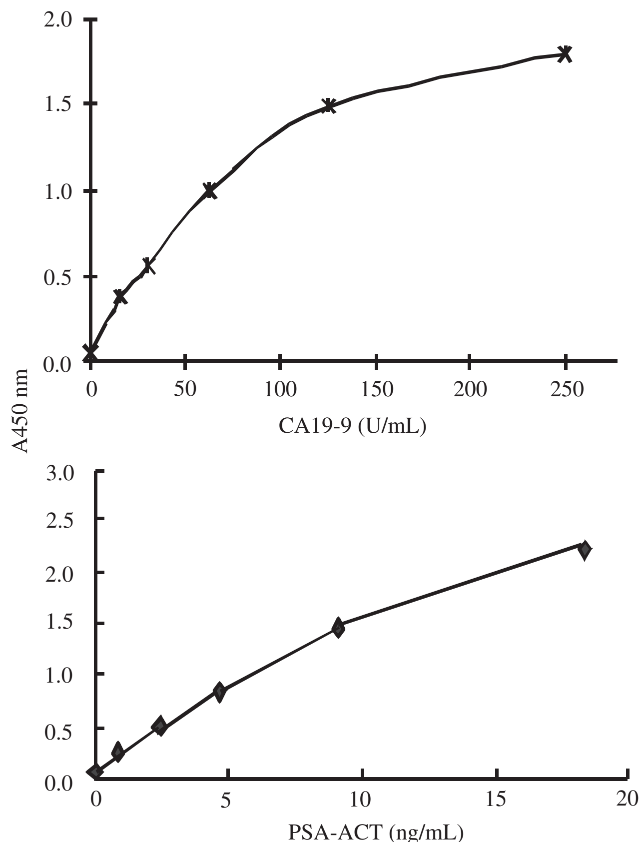

Fig. 4. Calibration curves of 384-well ELISA for CA 19-9 and PSA-ACT complex.

DISCUSSION

The major differences between 96- and 384-well microplates are summarized in Table 8. In addition to the difference in number of wells, they also differ in the volume and light path (height). Apparently, the smaller volume of the 384-well did not affect the assay precision to a significant extent. We also anticipate better precision as a result of using automatic instruments for coating, pipeting, washing, and plate reading. In the current study, we only tested the impact on assay by reducing the sample volume and reagent by two-fold. We believe that further reduction in sample volume and reagents are possible if better test formats are designed.

The higher sensitivity of 384-ELISA appears to be related to the higher ratio of well-height to well-volume of 384-well microplate. Compared with 96-well microplate, the shape of the 384-well results in longer light path and, consequently, higher assay sensitivity. It also means that to reach the same assay sensitivity, less concentration of specimen and reagents are needed with 384-well ELISA. Capable of using less reagents and less precious specimens is especially important for establishing assays for tumor markers. The expenses associated with the commercial antibodies for tumor markers can be prohibitive in assay establishment.

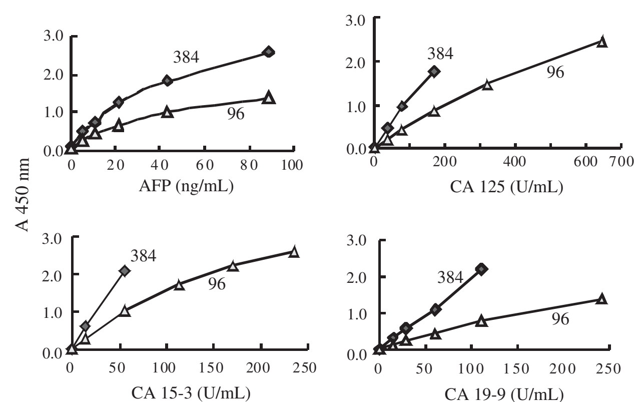

Fig. 5. Comparison between calibration curves of 384- and 96-well ELISA of the same tumor marker. Calibration curves of ELISA on 384-well (384) invariably exhibits higher absorbance and, thus, higher assay sensitivity using the same test format at the same concentration of samples.

TABLE 7. Within-day precision of ELISA performed on 96- and 384-well microplate

	AFP	CA 125	CA 15-3	CA 19-9
96-well (within-day)	24	24	24	24
Mean ± 2 SD	12.6 ± 0.4 (ng/mL)	57.5 ± 5 (U/mL)	27.9 ± 1 (U/mL)	36.1 ± 1.5 (U/mL)
(CV%)	(3.5)	(8.7)	(3.6)	(4.3)
Mean ± 2 SD	295 ± 8.7 (ng/mL)	104.7 ± 5.9 (U/mL)	122.2 ± 5 (U/mL)	119.1 ± 7.7 (U/mL)
(CV%)	(2.9)	(5.7)	(4.1)	(6.5%)
384-well (within-day)	15	15	15	15
Mean ± 2 SD	6.8 ± 1 (ng/mL)	17.4 ± 3 (U/mL)	22.0 ± 2.3 (U/mL)	14.2 ± 2 (U/mL)
(CV%)	(7.4)	(8.5)	(5.3)	(7.6)
Mean ± 2 SD	26.8 ± 4.5 (ng/mL)	220 ± 22.4 (U/mL)	73.7 ± 7.9 (U/mL)	66.7 ± 11.3 (U/mL)
(CV%)	(8.4)	(5.1)	(5.4)	(8.5)
384-well (day-to-day)	12	12	12	
Mean ± 2 SD	9.2 ± 1 (ng/mL)	20.4 ± 2 (U/mL)	24.0 ± 3 (U/mL)	
(CV%)	(9.2)	(9.5)	(8.2)	
Mean ± 2 SD	25 ± 5.2 (ng/mL)	150 ± 21.5 (U/mL)	82 ± 4.2 (U/mL)	
(CV%)	(9.8)	(8.9)	(10.1)	

TABLE 8. Major differences in wells between 96- and 384-well microplate

Number of well	384	96
Well height (light path)	0.5 cm (50 µl)	0.3 cm (100 µl)
Working volume	50 µl	100 µl
Total volume	120 µl	400 µl

There is another interesting phenomenon associated with the 384-well. There appears to be a more rapid diffusion of assay ingredients in the 384-well that results, in general, in less time during incubation for sample and antibody to reach equilibrium. This might have to do with the smaller diameter of the 384-well.

REFERENCES

1. Kassack MU, Hofgen B, Lehmann J, Eckstein N, Quillan JM, Sadee W. Functional screening of G protein-coupled receptors by measuring intracellular calcium with a fluorescence microplate reader. J Biomol Screen 2002;7:233–246.
2. Garrigues A, Nugier J, Orlowski S, Ezan E. A high-throughput screening microplate test for the interaction of drugs with p-glycoprotein. Anal Biochem 2002;305:106–114.
3. Wu JT. Quantitative immnoassay. A practical guide for assay establishment, troubleshooting, and clinical application. Washington, DC: AACC Press; 2000. p 1–225.