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High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format

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The oxygen radical absorbance capacity (ORAC) assay has been widely accepted as a standard tool to measure the antioxidant activity in the nutraceutical, pharmaceutical, and food industries. However, the ORAC assay has been criticized for a lack of accessibility due to the unavailability of the COBAS FARA II analyzer, an instrument discontinued by the manufacturer. In addition, the manual sample preparation is time-consuming and labor-intensive. The objective of this study was to develop a high-throughput instrument platform that can fully automate the ORAC assay procedure. The new instrument platform consists of a robotic eight-channel liquid handling system and a microplate fluorescence reader. By using the high-throughput platform, the efficiency of the assay is improved with at least a 10-fold increase in sample throughput over the current procedure. The mean of intra- and interday CVs was ≤15%, and the limit of detection and limit of quantitation were 5 and 6.25 μM, respectively.

KEYWORDS: ORAC; antioxidant activity; free radical; plate reader; high throughput

INTRODUCTION

Nutritionists, clinical researchers, and various segments of the food and pharmaceutical industries have an increasing need to know the antioxidant capacity of physiological fluids, foods, beverages, and natural products. This need is derived from the proven evidence of the importance of antioxidants to scavenge the reactive oxygen/nitrogen species (ROS/RNS), which are known to be involved in the pathogenesis of aging and many common diseases (*1*). Several methods for measuring antioxidant capacity in vitro have been developed and reviewed (*2*). The very complicated reaction among free radical, substrate, and antioxidant makes it impossible to use a fixed equation to express the kinetic order. Therefore, the accurate measurement of antioxidant capacity requires both inhibition degree and inhibition time to be taken into account. The oxygen radical absorbance capacity (ORAC) is the only method so far that combines both inhibition time and degree of inhibition into a single quantity (*3*). The early version of the ORAC assay developed by Cao et al. was time-consuming and labor-intensive, particularly for analyses of large numbers of samples (*4*). Later, the ORAC method was semiautomated by adapting it to a COBAS FARA II analyzer, an instrument discontinued by the manufacturer (*5*). This semiautomated ORAC can analyze up to nine samples at a single concentration per run. However, the reading of a single concentration usually does not fall within the linear concentration range of standards; thus, repeated runs are needed until a satisfactory result is obtained. Moreover, the long sample preparation times often consume most of a day, whereas the COBAS FARA II "waits" for sample. In addition to the low efficiency of sample throughput, the earlier version of ORAC has several limitations, including interactions between the fluorescent probe and tested antioxidants and incompatibility with lipid soluble antioxidants. Most recently, the ORAC assay was significantly improved by Ou, Huang, and co-workers using fluorescein as the new fluorescent probe (*6*−*7*). The improved ORAC was demonstrated to be robust and compatible with lipid soluble antioxidant. However, the new version of ORAC was developed on the COBAS FARA II platform and, as such, is available only in a few laboratories equipped with the COBAS FARA II. This situation not only severely limits access to the assay by other researchers but also causes low productivity. Therefore, development of a high-throughput instrument platform that fully automates the ORAC assay from sample preparation to final measurement is necessary. In the present study, we investigated the feasibility of full automation for the ORAC assay using a widely utilized platform consisting of a

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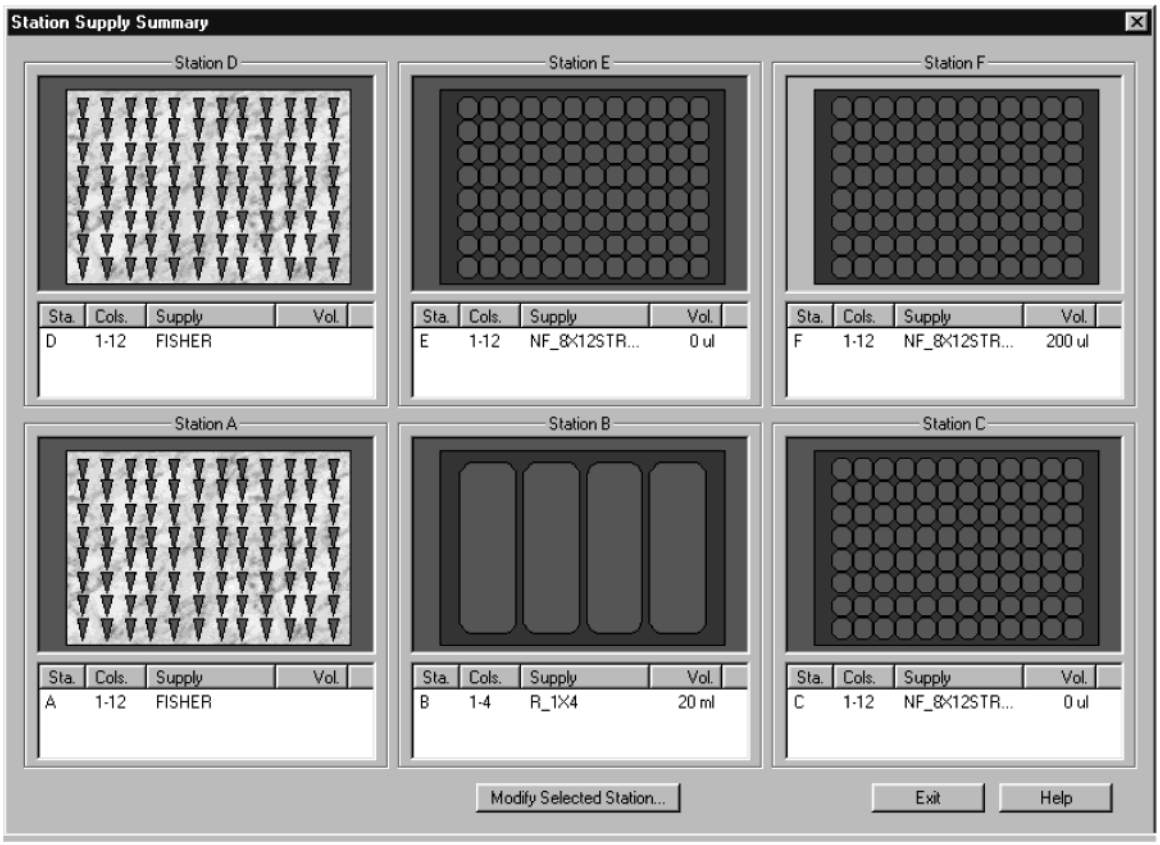


Figure 1. Layout of the deck of the Bio-Tek Precision 2000 showing the locations of stations.

multichannel liquid handling system on-line with a microplate fluorescence reader in 96-well format. Our results demonstrate that the new automation platform can analyze at least 130 samples per day without repeated runs, compared with the COBAS-based ORAC method, which would take one analyst at least several working days to complete. This high-throughput improvement not only completely eliminates human error in several steps of sample preparation and substantially shortens the assay time but also frees analysts for other research activities.

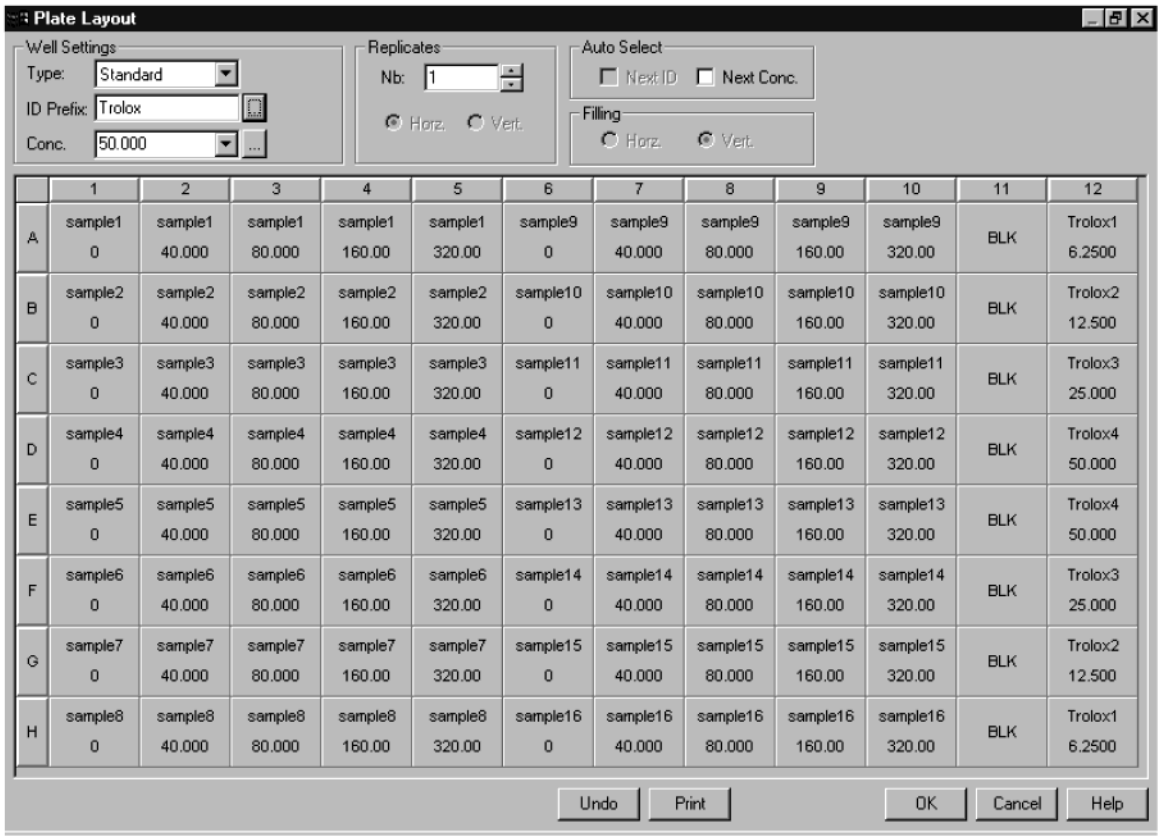
MATERIALS AND METHODS

Chemicals and Apparatus. Trolox and fluorescein disodium were obtained from Aldrich (Milwaukee, WI). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). B-Phycoerythrin (B-PE) and 15 phenolic compounds were obtained from Sigma Chemical Co. (St. Louis, MO). Coffee powder, rosemary extract, strawberry extract, and grape juice were obtained in-house. Plasma was withdrawn from three volunteers at Brunswick Laboratories. An FL600 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT) was used with fluorescence filters for an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm. The plate reader was controlled by software KC4 3.0 (revision 29). Sample dilution was accomplished by a Precision 2000 automatic pipetting system managed by precision power software (version 1.0) (Bio-Tek Instruments, Inc.). The 96-well polystyrene microplates and the covers were purchased from VWR International Inc (Bridgeport, NJ). A COBAS FARA II analyzer (Roche Diagnostic System Inc., Branchburg, NJ) was used for a comparison study.

Sample Preparation. Pure phenolic compounds were directly dissolved in an acetone/water mixture (50:50, v/v) and diluted with 75 mM potassium phosphate buffer (pH 7.4) for analysis. Coffee, rosemary extract, and strawberry extract were initially ground in a mechanical mill to produce a fine power. Then 0.5 g of the powders was accurately weighed, and 20 mL of acetone/water (50:50, v/v) extraction solvent was added. The mixture was shaken at 400 rpm at room temperature on an orbital shaker for 1 h. The extracts were centrifuged at 14000 rpm for 15 min, and the supernatant was ready for analysis after appropriate dilution with buffer solution. For grape juice, a 20 mL aliquot of sample was centrifuged for 15 min, and the supernatant was ready for analysis after appropriate dilution. Plasma was ready for analysis without further preparation.

Reagent and Standard Preparation. AAPH (0.414 g) was completely dissolved in 10 mL of 75 mM phosphate buffer (pH 7.4) to a final concentration of 153 mM and was kept in an ice bath. The unused AAPH solution was discarded within 8 h. Fluorescein stock solution (4.19×10^{-3} mM) was made in 75 mM phosphate buffer (pH 7.4) and was kept at 4 °C in dark condition. The fluorescein stock solution at such condition can last several months. The 8.16×10^{-5} mM fresh fluorescein working solution was made daily by further diluting the stock solution in 75 mM phosphate buffer (pH 7.4). Trolox standard was prepared as follows: 0.250 g of Trolox was dissolved in 50 mL of 75 mM phosphate buffer (pH 7.4) to give a 0.02 M stock solution. The stock solution was diluted with the same phosphate buffer to 50, 25, 12.5, and 6.25 μM working solutions.

Automated Sample Preparation. The automated sample preparation was performed using a Precision 2000. The layout of the deck of the Bio-Tek Precision 2000 is illustrated in **Figure 1**. As shown, the 250 μL pipet racks were placed at stations A and D. Station B was the reagent vessel in which 50 mL of 8.16×10^{-5} mM fluorescein was placed in reagent holder 1 and 50 mL of 75 mM phosphate buffer (pH 7.4) was added in reagent holder 2. A 96-well polypropylene plate (maximum well volume = 320 μL) was placed at station C for sample dilution. The initial addition of samples into the 96-well plate at station C was done by manual mode using an eight-channel pipet. Briefly, 200 μL of 75 mM phosphate buffer (blank) was dispensed into column 11 (wells A11−H11). The Trolox standard solution was added into column 12 (wells A12−H12) as follows: 6.25 μM (A12), 12.5 μM (B12), 25 μM (C12), 50 μM (D12), 50 μM (E12), 25 μM (F12), 12.5 μM (G12), and 6.25 μM (H12). Then eight samples were pipetted into column 1 (wells A1−H1) and column 6 (wells A6−H6), respectively. The sample series dilution sequence was programmed and controlled



	1	2	3	4	5	6	7	8	9	10	11	12
A	sample1 0	sample1 40.000	sample1 80.000	sample1 160.00	sample1 320.00	sample9 0	sample9 40.000	sample9 80.000	sample9 160.00	sample9 320.00	BLK	Trolox1 6.2500
B	sample2 0	sample2 40.000	sample2 80.000	sample2 160.00	sample2 320.00	sample10 0	sample10 40.000	sample10 80.000	sample10 160.00	sample10 320.00	BLK	Trolox2 12.500
C	sample3 0	sample3 40.000	sample3 80.000	sample3 160.00	sample3 320.00	sample11 0	sample11 40.000	sample11 80.000	sample11 160.00	sample11 320.00	BLK	Trolox3 25.000
D	sample4 0	sample4 40.000	sample4 80.000	sample4 160.00	sample4 320.00	sample12 0	sample12 40.000	sample12 80.000	sample12 160.00	sample12 320.00	BLK	Trolox4 50.000
E	sample5 0	sample5 40.000	sample5 80.000	sample5 160.00	sample5 320.00	sample13 0	sample13 40.000	sample13 80.000	sample13 160.00	sample13 320.00	BLK	Trolox4 50.000
F	sample6 0	sample6 40.000	sample6 80.000	sample6 160.00	sample6 320.00	sample14 0	sample14 40.000	sample14 80.000	sample14 160.00	sample14 320.00	BLK	Trolox3 25.000
G	sample7 0	sample7 40.000	sample7 80.000	sample7 160.00	sample7 320.00	sample15 0	sample15 40.000	sample15 80.000	sample15 160.00	sample15 320.00	BLK	Trolox2 12.500
H	sample8 0	sample8 40.000	sample8 80.000	sample8 160.00	sample8 320.00	sample16 0	sample16 40.000	sample16 80.000	sample16 160.00	sample16 320.00	BLK	Trolox1 6.2500

Figure 2. Layout of the 96-well microplate at station C. The number in each cell represents the sample dilution factor.

by the precision power software (version 1.0). An initial 1:40 dilution was performed followed by consecutive 1:2, 1:2, and 1:2 dilutions; this would give a series of 1:40, 1:80, 1:160, and 1:320 dilutions. Any other desired lower dilution can be obtained by simply performing a series of 1:4 or 1:8 dilutions after the initial 1:40 dilution. Care was taken to ensure homogeneity of each dilution by thorough mixing at each stage through repeated aspiration and dispensing programmed by the precision power software. There is no dilution needed for Trolox standards and blank. **Figure 2** illustrates the layout for the plate at station C.

Automated Reagent Addition. A second 96-well polystyrene plate was placed on station E. A full automation of plate to plate liquid transfer was programmed. Specifically, 150 μL of fluorescein working solution from reagent holder 1 at station B was transferred to column 1 through column 12 at station E. Then 25 μL of blank solution from column 11 at station C was transferred to columns 1 and 12 at station E followed by 25 μL of Trolox standard from column 12 at station C and from the column 2 at station E. For the diluted sample, 25 μL of diluted sample solution from columns 2−5 at station C was transferred to column 3−6 at station E. Similarly, 25 μL of diluted sample solution from columns 7−10 at station C was also transferred to the corresponding columns 7−10 at station E. Column 11 at station E was used for the control sample, to which 25 μL of 25 μM gallic acid was transferred from station F. The plate was covered with a lid and incubated in the preheated (37 °C) FL 600 microplate reader for 10 min with a 3 min shaking during this time. The plate was then transferred back to station E followed by the addition of 25 μL of AAPH solution transferred from reagent holder 3 at station B. Thus, the total volume for each well was 200 μL. The plate was immediately transferred to the plate reader, and the fluorescence was measured every minute for 35 min. The fluorescence readings are referenced to the highest reading of wells in column 1 or 12, in which no AAPH was added. **Figure 3** shows the layout of a 96-well plate used for measurement. Under this protocol, each sample generates four ORAC values at different concentrations, and the final ORAC result is expressed as the average of the four determinations.

Data Processing. ORAC values are calculated according to a previous paper (*3*). Briefly, the net area under the curve (AUC) of the standards and samples was calculated. The standard curve is obtained by plotting Trolox concentrations against the average net AUC of the two measurements for each concentration. Final ORAC values are calculated using the regression equation between Trolox concentration and the net AUC and are expressed as micromole Trolox equivalents per liter for liquid samples or per gram for solid samples. The AUC is calculated as

$$\mathrm{AUC} = 0.5 + f_1/f_0 + \ldots f_i/f_0 + \ldots + f_{34}/f_0 + 0.5(f_{35}/f_0) \quad (1)$$

where f_0 = initial fluorescence reading at 0 min and f_i = fluorescence reading at time i.

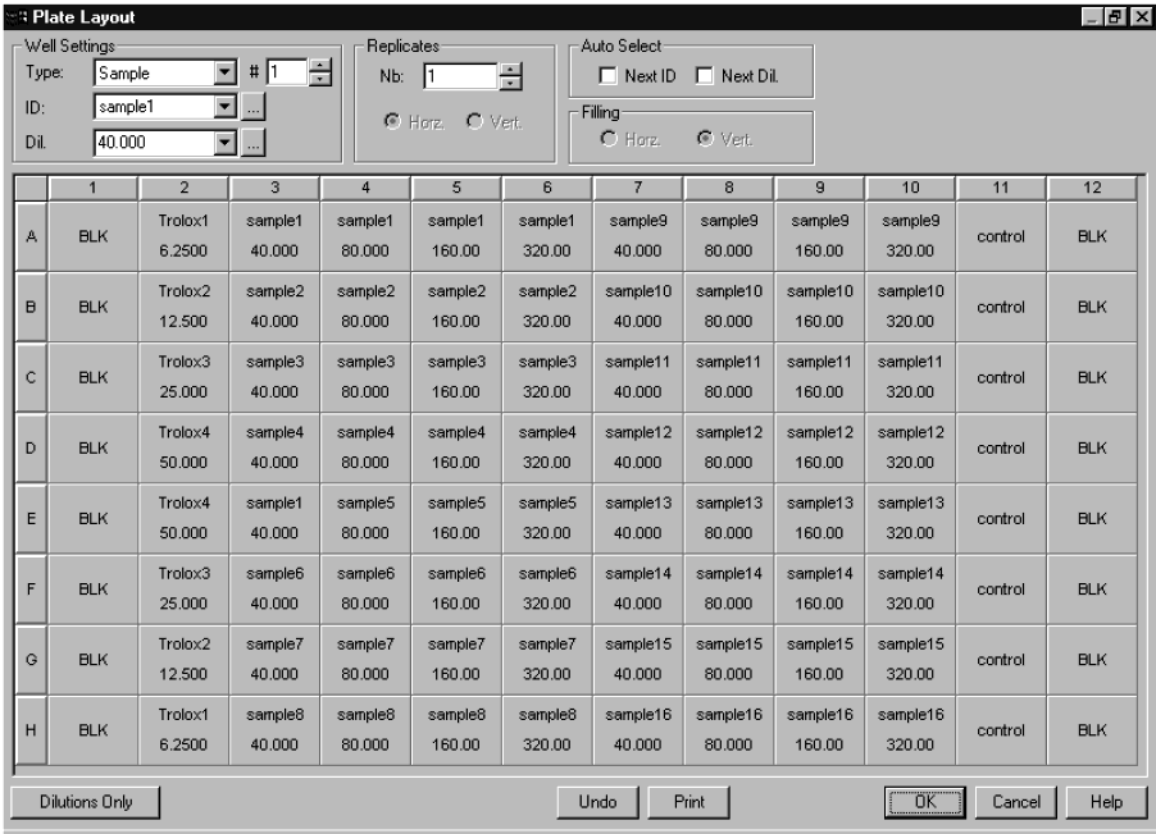
The data were analyzed by a Microsoft Excel macro program (Microsoft, Roselle, IL) to apply eq 1 to calculate the AUC. The net AUC is obtained by subtracting the AUC of the blank from that of a sample. The relative Trolox equivalent ORAC value is calculated as

$$\text{relative ORAC value} = [(\mathrm{AUC}_{\mathrm{sample}} - \mathrm{AUC}_{\mathrm{blank}})/(\mathrm{AUC}_{\mathrm{Trolox}} - \mathrm{AUC}_{\mathrm{blank}})](\text{molarity of Trolox/molarity of sample}) \quad (2)$$

RESULTS

Photostability of the Fluorescent Probe. In **Figure 4**, 200 μL of 8.16×10^{-5} mM fluorescein was exposed to excitation light at 491 nm in the absence of AAPH over a 35 min period. It is evident that there are no significant fluorescence intensity changes over 35 min; therefore, 8.16×10^{-5} mM fluorescein under such conditions is photostable. In contrast to fluorescein, B-PE, the fluorescent probe originally used in Cao's ORAC assay (*4*), was found to have lost almost 53% of its fluorescence intensity at the same conditions over 35 min. Apparently, B-PE is not suitable as a probe under the plate-reader conditions.

Linearity. The linear relationship between AUC and antioxidant concentration was evaluated using various pure compounds, natural products, beverages, and plasma at different concentrations. All analyzed samples in the various forms



	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	Trolox1 6.2500	sample1 40.000	sample1 80.000	sample1 160.00	sample1 320.00	sample9 40.000	sample9 80.000	sample9 160.00	sample9 320.00	control	BLK
B	BLK	Trolox2 12.500	sample2 40.000	sample2 80.000	sample2 160.00	sample2 320.00	sample10 40.000	sample10 80.000	sample10 160.00	sample10 320.00	control	BLK
C	BLK	Trolox3 25.000	sample3 40.000	sample3 80.000	sample3 160.00	sample3 320.00	sample11 40.000	sample11 80.000	sample11 160.00	sample11 320.00	control	BLK
D	BLK	Trolox4 50.000	sample4 40.000	sample4 80.000	sample4 160.00	sample4 320.00	sample12 40.000	sample12 80.000	sample12 160.00	sample12 320.00	control	BLK
E	BLK	Trolox4 50.000	sample1 40.000	sample5 80.000	sample5 160.00	sample5 320.00	sample13 40.000	sample13 80.000	sample13 160.00	sample13 320.00	control	BLK
F	BLK	Trolox3 25.000	sample6 40.000	sample6 80.000	sample6 160.00	sample6 320.00	sample14 40.000	sample14 80.000	sample14 160.00	sample14 320.00	control	BLK
G	BLK	Trolox2 12.500	sample7 40.000	sample7 80.000	sample7 160.00	sample7 320.00	sample15 40.000	sample15 80.000	sample15 160.00	sample15 320.00	control	BLK
H	BLK	Trolox1 6.2500	sample8 40.000	sample8 80.000	sample8 160.00	sample8 320.00	sample16 40.000	sample16 80.000	sample16 160.00	sample16 320.00	control	BLK

Figure 3. Layout of the 96-well microplate at station E. The number in each cell represents the sample dilution factor.

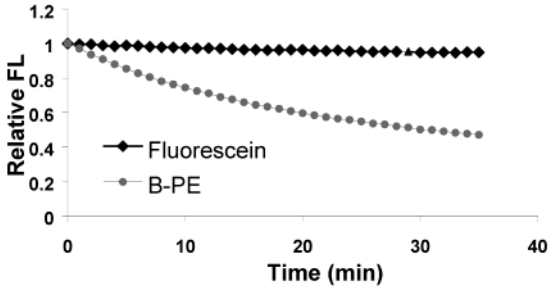


Figure 4. Fluorescence decay curves of B-PE and fluorescein in the absence of AAPH. $T = 37$ °C; data recorded on a Bio-Tek FL600 microplate fluorescence reader. As illustrated, the fluorescence intensity of B-PE lost almost 50% over 35 min, whereas no loss of fluorescence was observed for fluorescein.

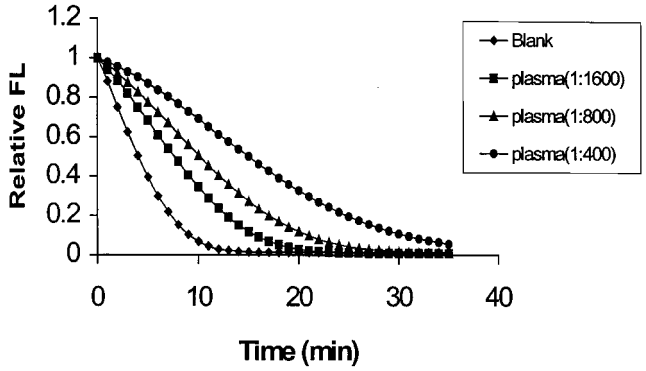


Figure 5. Fluorescence decay curves of fluorescein induced by AAPH in the presence of plasma diluted by pH 7.4 phosphate buffer.

demonstrate a good linear relationship between AUC and concentration. **Figure 5** illustrates the fluorescein fluorescence decay curves in the presence of plasma and AAPH. **Table 1** summarizes Trolox linear results: the average slope for Trolox is 1.492 ± 0.043, the correlation coefficient is >0.99, and the intercept ranges over -8.68 ± 1.54. The intercept is due to the AUC of the blank; we found that it does not affect the ORAC results. The linearity range for Trolox falls between 6.25 and 50 μM. The limit of quantitation (LOQ) and the limit of detection (LOD) are 6.25 and 5 μM (with AUC of $\sim$5), respectively, using Trolox as a calibration standard. **Table 2** shows the linear data for the analyzed samples.

Table 1. Linearity of Trolox Standard Curves

run	slope	intercept	correlation coefficient
1	1.5664	–10.644	0.9926
2	1.4929	–7.2126	0.9982
3	1.4688	–7.7400	0.9900
4	1.4991	–9.0339	0.9910
5	1.4666	–9.044	0.9900
6	1.5215	–10.453	0.9984
7	1.4314	–6.6994	0.9929
av	1.4923	–8.6895	0.9904
SD	0.0434	1.5393	0.0018
%RSD	2.9094	–17.714	0.1870

Precision. The 12 representative samples were tested over 5 days in duplicate each day. The standard deviation (SD) and coefficient of variation (%CV) of the ORAC values were calculated for intraday and interday. As shown in **Table 3**, both intraday and interday %CV values are $<15\%$.

Accuracy. Uric acid was chosen to evaluate the accuracy study, and the results are shown in **Table 4**. Uric acid at three different concentrations was tested, and the accuracy varies from 99 to 109% within individual batches and from 102 to 107% between all of the batches.

Ruggedness. We measured the ORAC value of quercetin (6.25 μM) over a 50 day period, and the net AUCs are plotted

Table 2. Linear Ranges of Various Samples

sample	concn range (μM)[a]	slope	intercept	R^2
EGC	2.00–16.00	1.5441	1.7942	0.9994
quercetin	1.00–15.00	4.5567	5.2845	0.9969
ferulic acid	1.00–12.00	2.2108	5.2906	0.990
EGCG	1.00–15.00	2.5137	2.1445	0.9980
strawberry extract	0.013–0.16 (g/L)	359.1	3.979	0.9970
coffee powder	3.10×10^{-4}–2.5×10^{-3} (g/L)	7761	3.4438	0.9955
rosemary extract	5.31×10^{-4}–4.25×10^{-3} (g/L)	8447.6	7.2011	0.9974
grape juice	3.13×10^{-4}–2.5×10^{-3} (v/v)	19431	5.6118	0.9943
plasma 1	6.25×10^{-5}–5×10^{-3} (v/v)	5664.3	5.7184	0.9912
plasma 2	6.25×10^{-5}–5×10^{-3} (v/v)	6001.5	6.5869	0.9948
plasma 3	6.25×10^{-5}–5×10^{-3} (v/v)	6231.9	5.8989	0.9965
caffeic acid	1.56–12.50	3.5255	8.0024	0.9910
hydroxybenzoic acid	1.56–12.50	2.0780	2.6860	0.9951
hydroxycinnamic acid	1.56–12.50	1.8427	2.4014	0.9967
2,4-dihydroxybenzoic acid	1.56–12.50	1.7391	3.7208	0.9904
kaempferol	1.56–12.50	2.1979	–0.0875	0.9951
2,3-dihydroxybenzoic acid	1.56–12.50	2.9200	6.2068	0.9966
myricetin	1.56–12.50	2.1661	12.0771	0.9928
ferulic acid	1.56–12.50	2.1388	4.9990	0.9928
protocatechuic acid	1.56–12.50	3.6307	7.2109	0.9952
quercetin	1.56–12.50	6.9713	4.9713	0.9986
gallic acid	1.56–12.50	0.9738	0.1405	0.9969

[a] Concentration given in μM except where noted otherwise.

Table 3. Reproducibility of the ORAC Assay Performed by the 96-Well Plate Reader ($n \geq 4$)[a]

	day 1			day 2			day 3			day 4			day 5			interday av		
sample	ORAC	SD	%CV	ORAC	SD	%CV	ORAC	SD	%CV	ORAC	SD	%CV	ORAC	SD	%CV	ORAC	SD	%CV
EGC	2.41	0.26	10.93	2.42	0.01	0.25	2.44	0.16	6.50	2.08	0.19	8.97	2.92	0.11	3.73	2.34	0.12	4.94
quercetin	7.51	0.45	5.97	6.42	0.03	0.41	7.42	0.03	0.37	6.92	0.06	0.87	6.97	0.05	0.72	7.06	0.15	2.07
ferulic acid	2.32	0.10	12.59	2.66	0.12	7.83	2.62	0.50	19.11	2.49	0.24	9.70	2.49	0.24	9.66	2.52	0.05	1.91
EGCG	3.51	0.34	9.75	3.29	0.46	14.14	3.84	0.52	13.64	3.43	0.37	10.89	3.40	0.16	4.61	3.51	0.16	4.66
strawberry extract	5.35×10^2	41	7.81	4.99×10^2	27	5.51	5.98×10^2	26	4.47	5.39×10^2	18	3.42	5.37×10^2	37	6.88	5.43×10^2	36	6.77
coffee powder	1.06×10^4	492	4.65	1.07×10^4	356	3.34	9.96×10^3	422	4.24	1.04×10^4	801	7.73	1.03×10^4	67	0.65	1.10×10^4	336	3.23
rosemary extract	1.47×10^4	634	4.32	1.49×10^4	613	4.13	1.34×10^4	152	1.14	1.44×10^4	860	5.96	1.45×10^4	417	2.88	1.43×10^4	614	4.28
grape juice	3.35×10^4	833	2.49	2.99×10^4	558	1.87	3.50×10^4	831	2.38	3.19×10^4	166	0.52	3.12×10^4	1058	3.38	3.26×10^4	1251	3.84
plasma 1	8.51×10^3	22	0.27	8.99×10^3	355	3.96	7.95×10^3	688	8.65	8.62×10^3	192	2.24	8.06×10^3	572	7.10	8.52×10^3	329	3.86
plasma 2	8.58×10^3	60	0.70	9.22×10^3	195	2.12	8.62×10^3	674	7.82	9.50×10^3	9.0	0.10	8.43×10^3	1048	12.43	8.98×10^3	117	1.31
plasma 3	1.01×10^4	171	1.69	1.03×10^4	625	6.08	9.51×10^3	850	10.45	9.96×10^3	410	4.13	9.02×10^3	1147	12.72	9.96×10^3	324	3.25

[a] ORAC results for grape juice and plasma are expressed as micromole Trolox equivalents per liter; ORAC results for ECG, quercetin, ferulic acid, and EGCG are expressed as Trolox equivalents; ORAC results for strawberry extract, coffee powder, and rosemary extract are expressed as micromole Trolox equivalents per gram.

against the day in **Figure 6**. The %CV of the average AUC is 5.75%; therefore, the assay shows a very good reliability.

Comparison with COBAS FARA II. Table 5 lists the ORAC values of a group of samples that have been tested in parallel using both the COBAS FARA II centrifugal analyzer and the plate reader. With the exception of the results for rosemary extract (16.97) and EGC (15.04), all other results obtained by the plate reader are comparable to those generated by the COBAS FARA II. In addition, the accuracy and ruggedness of the plate reader is comparable to those of the COBAS FARA II as the %CV is <15%. Therefore, we conclude that the ORAC assays on two different instrument systems give equally valid data.

DISCUSSION

Principle of the ORAC Assay. Various methods have been developed to characterize the total antioxidant capacity for biological fluids and natural products (*8*−*12*). Compared to other methods, the semiautomated ORAC protocol using the COBAS FARA II developed by Cao and Prior et al. has received extensive coverage and utilization in the field of antioxidant and oxidative stress (*13*−*17*). The ORAC assay depends on the free radical damage to a fluorescent probe through the change in its fluorescence intensity. The change of fluorescence intensity is an index of the degree of free radical damage. In the presence of antioxidant, the inhibition of free radical damage by an antioxidant, which is reflected in protection against the change of probe fluorescence in the ORAC assay, is a measure of its antioxidant capacity against the free radical (**Figure 7**). The uniqueness of the ORAC assay is that the reaction is driven to completion and the quantitation is achieved using "area under the curve" (AUC). In particular, the AUC technique allows ORAC to combine both inhibition time and inhibition percentage of the free radical damage by the antioxidant into a single quantity (**Figure 8**).

Limitation of the Original ORAC Assay. The ORAC assay has been criticized for a lack of accessibility due to unavailability of the COBAS FARA II, an instrument discontinued by the manufacturer. As mentioned before, only a few laboratories in North America are able to perform the ORAC assay, and this situation adversely affects widespread utilization of the ORAC assay. Although some efforts were made in the utilization of an alternative instrument, very little has been successful. For instance, Caldwell constructed a semiautomatic instrument for ORAC determination, but it also is not available commercially (*18*). Other researchers attempted to adapt the ORAC assay to

Table 4. Precision and Accuracy of Quality Control Sample (Uric Acid)

	70 μM	50 μM	30 μM
run 1			
intramean	69.53	51.75	30.02
SD	2.07	1.65	1.18
%CV	2.98	3.19	3.93
REC	99.33	103.50	100.06
n	3	3	3
run 2			
intramean	72.71	54.68	32.14
SD	0.61	0.58	0.41
%CV	0.84	1.07	1.27
REC	103.87	109.35	107.14
n	3	3	3
run 3			
intramean	72.62	53.76	31.35
SD	1.74	1.64	1.16
%CV	2.39	3.05	3.70
REC	103.74	107.51	104.48
n	3	3	3
intermean	71.62	53.39	31.17
SD	1.81	1.50	1.07
%CV	2.52	2.81	3.44
REC	102.31	106.79	103.89
n	9	9	9

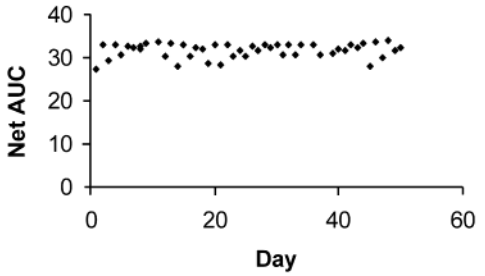


Figure 6. Ruggedness of ORAC method determined by 6.25 μM quercetin. The net AUC was obtained from a Bio-Tek FL600 fluorescence microplate reader over 50 days.

the microplate reader; however, B-PE was found to lose 30−50% of its intensity in the absence of free radical due to the photobleaching of B-PE (**Figure 4**). In addition to its photoinstability, B-PE was also observed to cause nonspecific protein binding with the analyzed compounds, in particular with

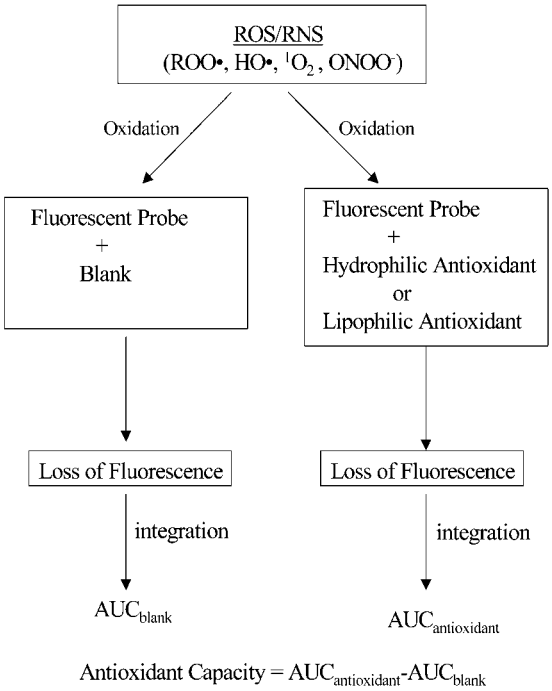


Figure 7. Schematic illustration of the principle of the ORAC assay.

flavonoids (*6*,*19*). The drawbacks of using of B-PE as the fluorescent probe prompted us to utilize a stable probe to replace B-PE. Recently, we reported the utilization of fluorescein as the new probe for the ORAC assay, and our results demonstrated that fluorescein is superior to B-PE in terms of photostability and reproducibility (*6*). However, our newly improved ORAC assay was developed on the COBAS FARA II platform; therefore, the issue of accessibility of the ORAC assay has not been addressed, and high sample throughput has not been achieved. In recent years, batch sample preparation using a liquid handling system coupled with a fluorescent microplate reader in 96-well format was becoming a common practice in clinical chemistry, biochemistry, and the pharmaceutical industry. The superior photostability of fluorescein makes it possible to transfer the ORAC methodology to this high-throughput platform.

Table 5. Comparison of ORAC Values Obtained by Bio-Tek FL 600 Fluorescence Plate Reader and COBAS FARA II Centrifugal Analyzer, Respectively ($n \geq 4$)[a]

sample	ORAC (plate reader)	ORAC (COBAS)	mean	%CV
EGC	2.34 ± 0.12	1.89 ± 0.13	2.12	15.04
ferulic acid	2.52 ± 0.05	2.54 ± 0.27	2.53	0.56
EGCG	3.51 ± 0.16	3.66 ± 0.04	3.59	2.96
strawberry extract	$(5.42 \pm 0.36) \times 10^2$	$(5.39 \pm 0.05) \times 10^4$	5.41×10^4	0.39
coffee powder	$(1.04 \pm 0.03) \times 10^4$	$1.28 \pm 0.23) \times 10^4$	1.16×10^4	14.63
rosemary extract	$(1.43 \pm 0.06) \times 10^4$	$(1.82 \pm 0.32) \times 10^4$	1.63×10^4	16.97
grape juice	$(3.26 \pm 0.13) \times 10^4$	$(3.37 \pm 0.04) \times 10^4$	3.32×10^4	2.35
plasma 1	$(8.52 \pm 0.33) \times 10^3$	$(9.13 \pm 0.23) \times 10^3$	8.83×10^3	4.89
plasma 2	$(8.98 \pm 0.12) \times 10^3$	$(1.08 \pm 0.22) \times 10^4$	9.89×10^3	13.01
plasma 3	$(9.96 \pm 0.32) \times 10^3$	$(9.46 \pm 0.19) \times 10^3$	9.71×10^3	3.64
hydroxybenzoic acid	2.38 ± 0.14	2.01 ± 0.16	2.20	11.92
hydroxycinnamic acid	2.16 ± 0.35	2.05 ± 0.06	2.11	3.70
2,4-dihydroxybenzoic acid	2.11 ± 0.12	1.72 ± 0.18	1.92	14.40
kaempferol	2.29 ± 0.11	2.75 ± 0.23	2.52	12.91
2,3-dihydroxybenzoic acid	4.45 ± 0.25	4.36 ± 0.41	4.41	1.44
myricetin	4.26 ± 0.63	4.64 ± 0.29	4.45	6.04
protocatechuic acid	5.14 ± 0.10	5.21 ± 0.16	5.18	0.96
quercetin	7.06 ± 0.15	6.46 ± 0.52	6.76	6.28

[a] ORAC results for grape juice and plasma are expressed as micromole Trolox equivalents per liter; ORAC results for pure chemicals are expressed as Trolox equivalents; ORAC results for strawberry extract, coffee powder, and rosemary extract are expressed as micromole Trolox equivalents per gram.

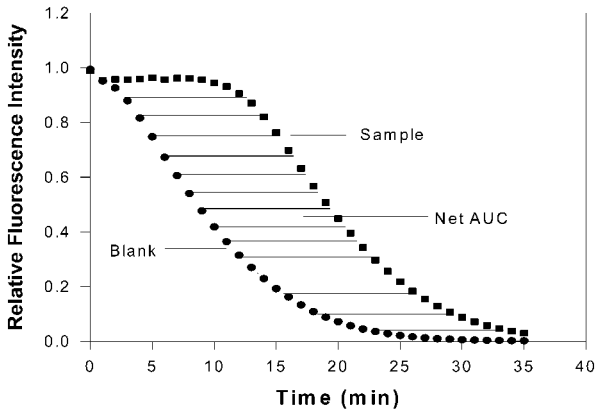


Figure 8. Illustration of calculation the ORAC value expressed as the net area under the curve (AUC). For calculation of the AUC, please see eq 2 in the text.

Automated Sample Preparation. The improved ORAC assay is extremely sensitive; thus, the sample usually needs to be diluted at least 50- to several hundred-fold prior to the analysis. Unfortunately, the traditional manual sample preparation has been time-consuming and labor-intensive, requiring dilutions to be performed manually, in addition to labeling of tubes and vials and the sequential sample processing. Therefore, the sample preparation step became a bottleneck in the development of high-throughput ORAC analysis. The Precision 2000 has a completely configurable six-station platform to hold the required pipet tips, reagent troughs, and microplates (96- and 384-well) for fluid transfer. The eight-channel pipet arm moves up and down as well as side-to-side, while the platform moves front to back to provide complete access to all locations on the work platform and complete configurability. As a result, all liquid-transfer steps including series dilution, addition of fluorescent probe and free radical initiator, and transfer of analyzed samples are completely automated. The steps previously performed manually can now be prepared in parallel using the eight-channel pipet with a total cycle time for sample preparation of a 96-well plate, consisting of 16 samples with 4 series of dilution for each sample, 4 standards in duplication, and 8 blanks, of only 15 min. A 35 min cycle time per 96-well plate is currently routine for the FL 600 plate reader, resulting in a throughput of at least 130 samples per day. The reagent holders containing buffer and fluorescein working solution need to be filled at the start of the assay, and the only manual intervention required is loading of the 96-well plates containing samples, AAPH, and QC samples to the their corresponding stations in the Precision 2000 liquid handling system.

Temperature Issue of the 96-Well Plate. Temperature plays a critical role in the ORAC assay, because the reaction is initialized by the thermal decomposition of AAPH. However, due to the poor thermal conductivity of the polypropylene plate, possible temperature inhomogeneity may occur from well to well, causing considerable variations in the AUC. To eliminate this problem, we preheat the plate at 37 °C for 10 min before the addition of AAPH. The addition of AAPH takes only <1 min and therefore does not affect the temperature significantly.

In summary, the eight-channel robotic liquid handler coupled with a 96-well plate reader provides substantial advantages over the current semiautomatic procedure. The efficiency is improved with at least a 10-fold increase in sample preparation and in instrument utilization over the current procedure, to at least 130 samples per day. The use of the robotic liquid handling and plate reader technologies has not compromised the quality of data obtained. Instead, human errors and frustration associated with the tedious sample preparation have been completely eliminated, resulting in more accurate and precise data. The high-throughput platform described in this paper can also be utilized to perform the lipophilic ORAC assay developed by Huang and co-workers (*7*). This full automation would allow other researchers, especially biochemists, nutritionists, clinical researchers, and food chemists, to efficiently utilize the ORAC assay to study the oxidative stress and role of antioxidant in preventing the diseases induced by ROS/RNS.

ABBREVIATIONS USED

ORAC, oxygen radical absorbance capacity; AUC, area under the curve; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; B-PE, B-phycoerythrin; ROS, reactive oxygen species; RNS, reactive nitrogen species; EGC, epigallocatechin; EGCG, epigallocatechin gallate.

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