

## Combined Measurement of Ferritin, Soluble Transferrin Receptor, Retinol Binding Protein, and C-Reactive Protein by an Inexpensive, Sensitive, and Simple Sandwich Enzyme-Linked Immunosorbent Assay Technique<sup>1</sup>

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**ABSTRACT** The measurement of vitamin A (VA) and iron status is very important in the assessment of nutritional deficiencies. The objective of this research was to develop a sandwich ELISA technique for the simultaneous measurement of ferritin, soluble transferrin receptor, retinol binding protein, and C-reactive protein (CRP) as indicators for VA and iron status. The inclusion of CRP as marker of infection allows for more accurate interpretation of VA and iron status. This is accomplished in a 30- $\mu$ L serum or plasma sample using an ELISA with different capture and detection antibodies and different dilutions of the sample. Commercially available clinical serum controls were used for calibration purposes. The developed assays were compared to commercially available traditional tests. Regression coefficients comparing both assays were better than 0.84 ( $P < 0.001$ ). Using a limited sample set, the sandwich ELISA assay produced very similar specificity and sensitivity compared to traditional methods when common cutoff values were applied. Intra- and interassay variability was between 5 and 14% for all tests. The cost of the materials for all 5 measurements decreases to less than \$1/sample if a large number of samples is analyzed. Due to the low cost, high throughput, and comparability to traditional tests, this procedure has several advantages for assessing VA and iron status in population surveys. *J. Nutr.* 134: 3127–3132, 2004.

**KEY WORDS:** • ELISA • iron deficiency • vitamin A deficiency • low-cost method • infectious status

Vitamin A deficiency (VAD)<sup>3</sup> and iron deficiency (ID) are 2 of the most important micronutrient deficiencies worldwide. VAD results in >250,000 blind children every year and is responsible for increased morbidity and mortality in children and mothers throughout the poor populations of the nonindustrialized world (1). ID is even more prevalent, with an estimated 2 billion people affected. ID leads to reduced physical activity in adults and impaired brain development in children (2). To identify populations at risk for these deficiencies there is an urgent need for simple, reliable, and inexpensive methods to assess vitamin A (VA) and iron status. Because certain proteins can serve as markers of VA and iron status, we selected the sandwich ELISA technique for testing. It is a sensitive and fairly rugged method and uses inexpensive equipment and chemicals.

The 2 best parameters to assess iron status are ferritin and

soluble transferrin receptor (sTfR) (3). Ferritin correlates very well with iron stores and sTfR is increased as a result of ID. Using both values together it is even possible to calculate body iron stores, previously only possible with bone marrow staining, the “gold standard” for defining iron deficiency.

Nearly all vitamin A in the blood is associated with retinol binding protein (RBP). Therefore serum RBP can be used as a surrogate measure for retinol content and, thus, VA status (4). Levels of RBP correlate well with the more expensive and technically challenging HPLC measurement of serum retinol, the method recommended by WHO for assessing VA status (5).

Because ferritin levels are increased and RBP levels are lowered by infection, it is important to identify subjects with infection (6–8). For this purpose C-reactive protein (CRP) is a good measure of acute infections (7,9). Patients with elevated CRP should be excluded from evaluation or values corrected. If not, the results obtained for iron and VA markers could lead to a false-low rate of ID and a false-high rate of VAD.

A combined and optimized sandwich ELISA technique is not available for all these measurements, especially in a format using a single small-volume serum sample. Therefore the primary aim of this study was to optimize a simple sandwich

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<sup>3</sup> Abbreviations used: CRP, C-reactive protein; HRP, horseradish peroxidase; ID, iron deficiency; QC, quality control; RBP, retinol binding protein; sTfR, soluble transferrin receptor; TMB, 3,3',5,5'-tetramethylbenzidine dihydrochloride; VA, vitamin A; VAD, vitamin A deficiency.

ELISA method for each indicator to perform these measurements simultaneously increasing the method efficiency and providing a more comprehensive picture of the VA and iron status.

## MATERIALS AND METHODS

### Chemicals and other materials

The following chemicals were used as purchased:  $\text{NaH}_2\text{PO}_4$ ,  $\text{Na}_2\text{HPO}_4$ , NaCl, citric acid, phosphoric acid, 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB), 30%  $\text{H}_2\text{O}_2$  (Sigma).

**Capture antibodies.** Capture antibodies were as follows: ferritin (Code A0133, Dako), sTfR (Cat. No. 4Tr26; Clone 23D10, Hytest), RBP (Code A0040, Dako), CRP (Code A0073, Dako Denmark).

**Detection antibodies.** Detection antibodies were as follows: anti-ferritin-horseradish peroxidase (HRP) (Code P0145, Dako), anti-sTfR-HRP (Cat. No. 4Tr26-c; Clone 13E4, Hytest), anti-RBP-HRP (Code P0304, Dako), anti-CRP-HRP (Code P0227, Dako).

Serum control samples (Liquicheck, Bio-Rad) were used as standards for the calibration curves. Quality control (QC) samples were prepared from serum samples with a low and high content of analytes.

### ELISA procedure

**Coating 96-well plates with capture antibody.** Antibodies were diluted in coating buffer (0.01 mol/L phosphate buffer, 0.15 mol/L NaCl, pH 7.2). To 96-well plates (Maxi sorb C-shape, Nunc) was added 100  $\mu\text{L}$  of appropriately diluted antibody. The plates were covered with parafilm and were incubated overnight in the refrigerator. The antibody concentrations for coating were anti-ferritin, 0.05  $\mu\text{g}/\text{well}$  (1:5000); anti-sTfR, 0.1  $\mu\text{g}/\text{well}$  (1:10000); anti-RBP, 0.82  $\mu\text{g}/\text{well}$  (1:1000); and anti-CRP, 0.05  $\mu\text{g}/\text{well}$  (1:20,000).

The following morning the plates were emptied by inversion over a sink and prewashed by pouring wash buffer (0.01 mol/L phosphate buffer, pH 7.2, 0.5 mol/L NaCl, 0.1% Tween 20) over the plate and then slinging the buffer out into a sink. This was repeated 3 times, each time leaving the wash buffer 3–5 min in the wells. After the last wash any remaining wash buffer was removed by tapping the inverted plate on a paper towel.

**Application of serum and standards on the plate.** After the coated plates were washed, 100  $\mu\text{L}$  diluted serum and standard samples were added to the wells.

It is essential to place the patient samples and calibration standards on the plates in a way that minimizes biased readings to obtain reliable results. Therefore the measurement was done in duplicate and the 2 replicate samples were placed in different positions on the plate. The same procedure was done for the standards. A typical example for the arrangement of the samples on a plate is shown in **Figure 1**. A copy of this figure was also placed under the 96-well plate, which helps in finding the correct well during pipetting.

**Dilution scheme for serum.** The serum dilution scheme was as follows: D1, 15  $\mu\text{L}$  serum + 150  $\mu\text{L}$  wash buffer (1:11 dilution performed in 1.7-mL microtubes); D2, 10  $\mu\text{L}$  D1 + 1500  $\mu\text{L}$  wash

buffer (1:1661 dilution performed in 1.7-mL microtubes or a 96-deep-well plate).

**Volumes of diluted serum applied to the final reaction plate.** The following volumes were applied to the final reaction plate: ferritin, 100  $\mu\text{L}$  D1 (1:11 final dilution); sTfR, 100  $\mu\text{L}$  D2 (1:1661 final dilution); RBP: 25  $\mu\text{L}$  D2 (1:6644 final dilution); CRP: 50  $\mu\text{L}$  D2 (1:3322 final dilution).

For sTfR, RBP, and CRP the diluted serum was applied using a 12-channel pipette from the 96-deep-well plate or a plate that was filled with the D2 dilution of the samples. Before addition of the diluted serum to the plate, the wells for RBP and CRP were filled with the necessary amount of wash buffer to obtain a final volume of 100  $\mu\text{L}$ .

**Preparation of standards.** A commercially available control sample from Bio-Rad (Liquicheck Immunology Control, Level 3) was used to obtain a calibration curve on each plate. The manufacturer provides values for many analytes and methods of analysis. We calculated the mean value of all methods mentioned for each analyte of interest and used this value as the basis for further dilutions to obtain a calibration curve. The values were 296.9  $\mu\text{g}/\text{L}$  for ferritin, 2.87  $\mu\text{mol}/\text{L}$  for RBP, and 54.33 mg/L for CRP. Because no values were available for sTfR, we used a commercially available kit (Ramco Laboratories) to measure the concentration of sTfR (8.93 mg/L). To get calibration curves in the physiologically most interesting range we used the dilution scheme of **Table 1**.

After 2-h incubation at room temperature, the plate washing procedure was repeated as described above at the coating step. If a large number of samples is measured timing is critical: the samples and the standards should be incubated for similar amounts of time.

**Detection antibody binding.** A total of 100  $\mu\text{L}$  of diluted HRP coupled antibodies in coating buffer was added to the wells. The detection antibody concentrations were anti-ferritin-HRP, 0.015  $\mu\text{g}/\text{well}$  (1:8000); anti-sTfR-HRP, 0.015  $\mu\text{g}/\text{well}$  (1:15000); anti-RBP-HRP, 0.06  $\mu\text{g}/\text{well}$  (1:2000); and anti-CRP-HRP, 0.016  $\mu\text{g}/\text{well}$  (1:4000).

The plates were again incubated for 1 h at room temperature and the standard washing step was repeated.

**Color reagent and plate development.** To prepare the color reagent, 1 mg TMB (predissolved in DMSO) was added to 12 mL 0.1 mol/L citric acid phosphate buffer (pH 5.2). The TMB citric acid buffer solution can be prepared in larger amounts and stored frozen in 12-mL portions. To prevent border effects, it is essential that the plate and the color reagent are both at room temperature. Before the addition of 100  $\mu\text{L}$  of this color reagent to each well 2  $\mu\text{L}$  of 30%  $\text{H}_2\text{O}_2$  was mixed in to the 12-mL TMB solution. After sufficient blue color development (~5–10 min) the reaction was stopped by the addition of 100  $\mu\text{L}/\text{well}$  of 1 mol/L phosphoric acid. Each well was measured at 450 nm with the reference wavelength set at 650 nm. Although it is possible to measure without a reference wavelength the use of this reference wavelength can improve the quality of the measurement.

### Optimization of serum dilution, antibody concentration, and other tests

To make the measurement more economical, we reduced the antibody concentration (starting from 1  $\mu\text{g}/\text{well}$ ) and checked different serum dilutions. We selected the dilution that had a good regression coefficient for the standard curve and gave an appropriate color reaction within 10 min.

To evaluate the washing procedure, longer incubation with the wash buffer, higher Tween concentration, and an automatic washer were tested. None of these changes improved the CV or the background of the measurement. We also did not see any effect of other brands of plates specifically designed for ELISA tests. Blocking of the plates with an agent like albumin, dried skim milk, or gelatin usually resulted in a higher CV. The content of Tween in the wash buffer seemed high enough to prevent nonspecific reactions. We saw a positive effect of a blocking procedure in the sTfR assay, but only when the water quality was poor. Bacterial proteins might interfere with the sTfR assay if no blocking step is applied. PBS and bicarbonate buffers were equivalent for the capture antibody coating step.

	1	2	3	4	5	6	7	8	9	10	11	12
A	01	02	03	04	05	06	07	08	09	10	11	12
B	BL	L1	L2	L3	L4	L5	BL	L1	L2	L3	L4	L5
C	13	14	15	16	17	18	19	20	21	22	23	24
D	25	26	27	28	29	30	31	32	33	34	35	36
E	37	38	39	40	QC1	QC2	01	02	03	04	05	06
F	07	08	09	10	11	12	13	14	15	16	17	18
G	19	20	21	22	23	24	25	26	27	28	29	30
H	31	32	33	34	35	36	37	38	39	40	QC1	QC2

**FIGURE 1** Typical example for the arrangement of the samples on a 96-well plate (01–40: patient samples; BL, L1–L5: standards, QC1 and QC2 quality controls).

TABLE 1

*Dilution of Liquicheck Bio-Rad control sample for the preparation of calibration curves*

	Ferritin		sTfR		RBP		CRP	
	SD1, <sup>1</sup> $\mu\text{L}$	$\mu\text{g/L}^2$	SD2, <sup>3</sup> $\mu\text{L}$	$\text{mg/L}^2$	SD3, <sup>4</sup> $\mu\text{L}$	$\mu\text{mol/L}^2$	SD4, <sup>5</sup> $\mu\text{L}$	$\text{mg/L}^2$
Level 1	3	8.9	20	3.55	3	0.34	6	0.59
Level 2	6	17.8	40	7.10	6	0.69	12	1.18
Level 3	12	35.6	60	10.65	9	1.03	25	2.47
Level 4	25	74.2	80	14.19	12	1.38	50	4.94
Level 5	50	148.5	100	17.74	15	1.72	100	9.88

<sup>1</sup> SD1: 15  $\mu\text{L}$  Liquichek Immunology Control (Level 3) + 150  $\mu\text{L}$  wash buffer.

<sup>2</sup> Resulting concentration after filling up the wells to 100  $\mu\text{L}$ .

<sup>3</sup> SD2: 20  $\mu\text{L}$  SD1 + 1500  $\mu\text{L}$  wash buffer.

<sup>4</sup> SD3: 10  $\mu\text{L}$  SD1 + 1500  $\mu\text{L}$  wash buffer.

<sup>5</sup> SD4: 100  $\mu\text{L}$  SD3 + 1000  $\mu\text{L}$  wash buffer.

Limited experiments showed that undiluted serum samples could be thawed and refrozen a few times without any change in analyte concentration. Diluted serum samples also appear to be stable to freeze-thawing. Only sTfR seems to be altered by freeze-thawing. Diluted serum is, however, stable for 1 d at room temperature.

**Application studies.** To test this new procedure, convenience samples from various nutrition studies in developing countries that had been analyzed with traditional methods were analyzed with the sandwich ELISA assay. The correlation and agreement between the 2 methods was calculated. The specificity of the new method versus the traditional method using common cutoff values was also calculated. The following traditional methods were used as a reference point to compare the sandwich ELISA results: ferritin, RIA from Bio-Rad Laboratories; sTfR, ELISA from Ramco Laboratories; and RBP, retinol by HPLC-UV detection. For a limited number of samples we also investigated the agreement of the RBP values with a commercial RBP ELISA kit from ALPCO (Windham) and the CRP values with a CRP ELISA kit from IBL.

**Statistical procedures.** All analyses were done with Excel 2002 (Microsoft) and SPSS 11. The  $R^2$  value (square of the Pearson correlation coefficient) was calculated to examine the association between the traditional methods and the sandwich ELISA technique and the line of regression was tested against the line of identity (slope = 1, intercept = 0). A paired  $t$  test was used to test the difference between samples measured by the traditional method and the sandwich ELISA. Differences with  $P < 0.05$  were considered significant. To determine the agreement between 2 methods Bland-Altman bias analysis (10) was performed for the paired samples.

## RESULTS

The intra-assay variability for 5 replicates analyzed in the same assay was between 5.6 and 8.5%, with the lowest for sTfR and the highest for CRP. The interassay variability for analysis over 8 d was between 7.5 and 14.3%, again with the lowest for sTfR and the highest for CRP (Table 2).

By using the best fit function, the correlation coefficients of the standard curves were always better than 0.98. Differences between duplicate measurements on the same plate were  $< 10\%$ .

The best correlations between the sandwich ELISA assay and the traditional methods were obtained for ferritin and CRP (Fig. 2). The next best correlation was obtained for RBP compared to serum retinol and the weakest correlation was obtained for sTfR (Fig. 2).

To assess the agreement between the sandwich ELISA assay and traditional assays for ferritin, sTfR, RBP, and CRP, we used Bland-Altman bias plots (Fig. 3). Except for RBP, which had a small bias of 0.06  $\mu\text{mol/L}$ , there was no bias for the other proteins.

The RBP ELISA was additionally compared to a commercial RBP ELISA kit from ALPCO. The comparison of these 2 ELISAs showed good agreement: RBP (sandwich ELISA) =  $0.883 \times \text{RBP (ALPCO)} + 0.101$ ,  $R^2 = 0.78$ ,  $P < 0.001$ ,  $n = 16$ ).

The variances for the new method and the traditional method are very similar and the means of the new method versus the traditional method are not different (Table 3). By applying a cutoff value of 20  $\mu\text{g/L}$  for ferritin, 10 vs. 9 of 41 subjects were classified as iron deficient with the RIA vs. the sandwich ELISA (9 correct by both assays). Using a cutoff value of 8.3  $\text{mg/L}$  for sTfR, 79 vs. 85 of 119 subjects were classified as iron deficient with the Ramco ELISA versus the sandwich ELISA (76 correct by both assays). Using a cutoff value of 0.7  $\mu\text{mol/L}$  for retinol, 8 vs. 6 of 24 subjects were classified as vitamin A deficient with the HPLC versus the sandwich ELISA (6 correct by both assays). Using a cutoff value of 10  $\text{mg/L}$  for CRP, 9 vs. 9 of 17 subjects were classified as subjects having an infection with the IBL CRP ELISA kit vs. the sandwich ELISA (9 correct by both assays) (Table 3).

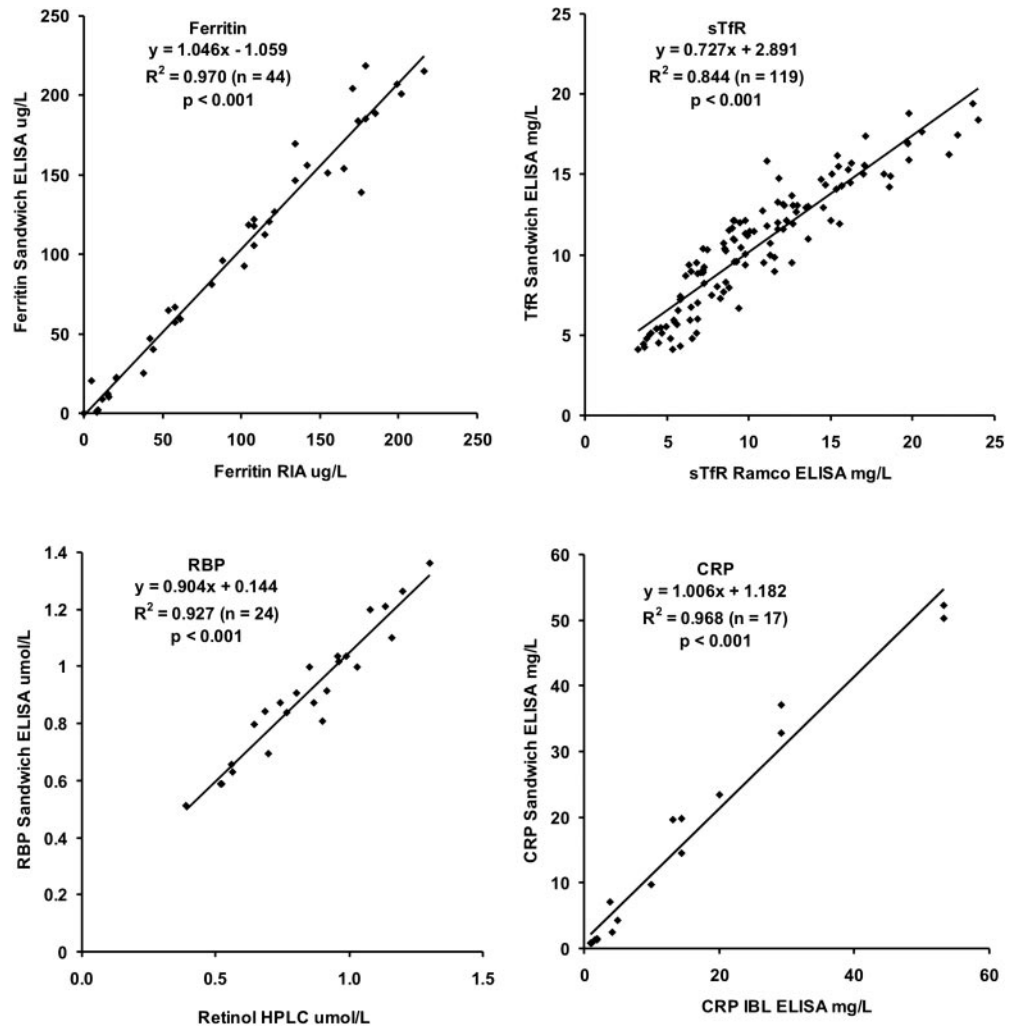
## DISCUSSION

In this paper we describe simple and inexpensive sandwich ELISA techniques to measure the VA and iron status together in 1 small serum sample. In addition, CRP as an indicator for acute infection can be measured using this technique. The latter is important because both VA and iron status indicators are altered during infection. The principle of this method is to coat 96-well plates with antibodies that "capture" the antigens in diluted serum samples or standards, and then after 2 h a detection antibody is added that is coupled to a peroxidase.

TABLE 2

*Intra- and interassay variability (% CV) for the sandwich ELISA methods for a control sample with a concentration of the measured proteins in a medium range*

	Intra-assay variability, CV % ( $n = 5$ )	Interassay variability, CV % ( $n = 8$ )
Ferritin	6.2	11.4
sTfR	5.6	7.5
RBP	7.3	12.9
CRP	8.5	14.3



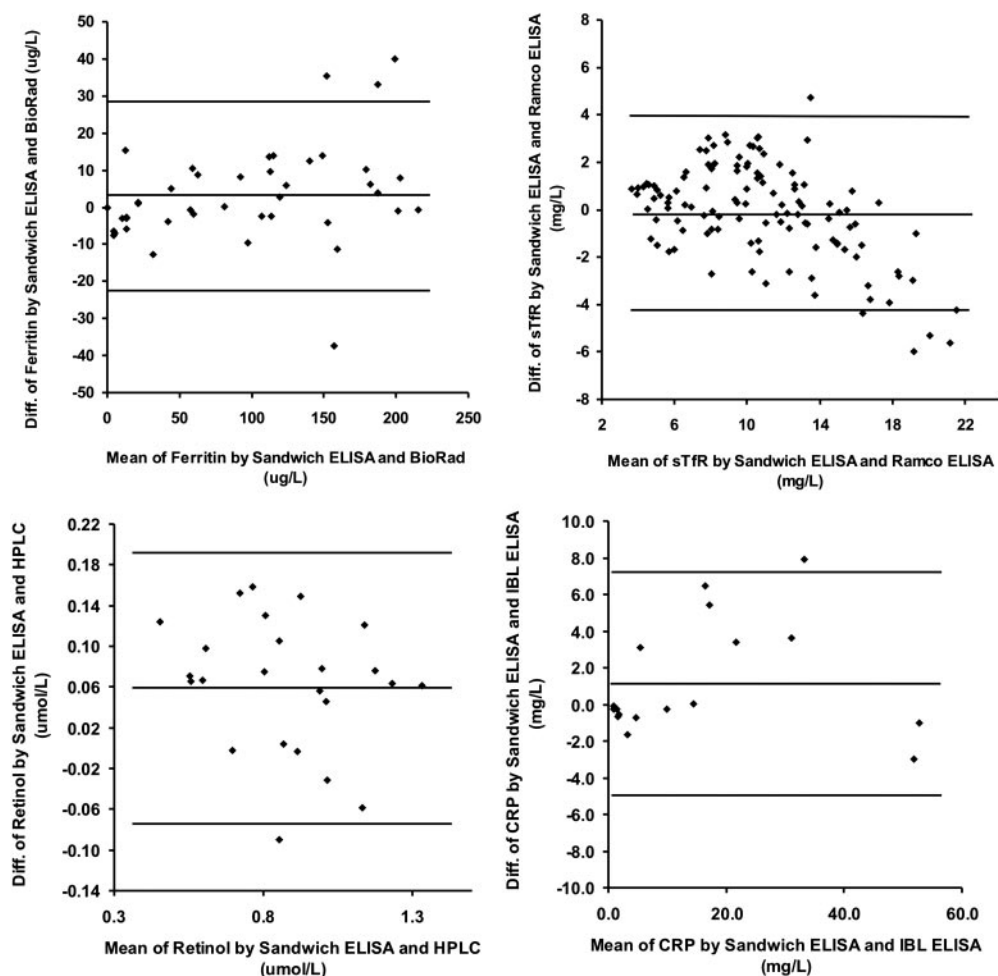
**FIGURE 2** Correlation plots for ferritin, sTfR, RBP, and CRP between the sandwich ELISA technique and the reference methods (Bio-Rad ferritin RIA, Ramco sTfR ELISA, retinol by HPLC, and IBL CRP ELISA).

The plates are washed before each application. The color intensity that develops after the addition of the color reagent is directly proportional to the amount of antigen in the sample.

The main points of merit presented for the sandwich ELISA technique include good intra-assay variability and acceptable interassay variability of ~10%. Moreover, the correlations and agreements obtained between the sandwich ELISA assay and traditional methods were very good. We found no bias between the sandwich ELISA and the Bio-Rad RIA assay for ferritin nor was there any between the sandwich ELISA and the Ramco ELISA assay for sTfR or the sandwich ELISA for CRP and the IBL CRP kit. We found a very small positive bias (0.06  $\mu\text{mol/L}$ ) between the sandwich ELISA assay for RBP and the HPLC method for retinol. Although the majority of samples analyzed for ferritin showed only moderate difference between the 2 methods tested, ~10% of the samples showed a much larger difference, which in turn resulted in a large variance of the mean difference. However, when we tested the means of the 2 methods for difference all 4 indicators produced method means that were not different. Moreover, the variances of the means for the 2 methods were also very comparable. Although the sample sets tested were certainly not large and did not contain many samples that would classify as iron or vitamin A deficient, the sandwich ELISA assay showed a very similar specificity compared to the traditional method.

To the best of our knowledge, the self-made sandwich ELISA technique is not widely used in nutrition laboratories although it has clear advantages with respect to cost, sensitivity, and throughput. In comparison to other immunological methods it is also fairly rugged. The main reason for this underutilization seems to be lack of experience and good standardization procedures, which are essential for obtaining reliable results. Instead, expensive ELISA kits or autoanalyzer methods are used, for which the costs of the materials are around \$5–10/measurement. If only a limited number of samples is analyzed, the cost of the assay is not such a critical factor; however, for large studies or surveys assay costs often make the measurement of these analytes prohibitive. The cost for the materials in the method presented is <20 cents/measurement, and the only relatively costly items are the antibodies. Each analyte requires a pair of antibodies that costs ~\$500 and is generally sufficient for >10,000 measurements.

Another advantage of the sandwich ELISA technique is high sensitivity. This makes it possible to perform all 5 measurements of interest in duplicate using 30  $\mu\text{L}$  of serum. Thus capillary blood from fingerstick sampling can be used. This is an important aspect, because capillary blood sampling is usually better accepted culturally and easier to perform, especially in children. The ferritin assay requires the largest amount of serum. If the volume of available serum is very limited, it is possible to further reduce the amount of serum by using a



**FIGURE 3** Bland-Altman bias plots for ferritin, sTfR, RBP, and CRP between the sandwich ELISA technique and the reference methods (Bio-Rad ferritin RIA, Ramco sTfR ELISA, retinol by HPLC, and IBL CRP ELISA, dotted lines  $\pm 2$  SD).

higher dilution for the ferritin assay. With this modification as little as 10  $\mu$ L of serum can be used to perform all tests.

The use of 96-well plates and a simple 12-channel pipette allows high throughput of samples. Forty samples for all 4 parameters or 4 plates with 160 samples for 1 parameter can usually be measured per day by a well-trained laboratory technician.

The approach of using commercially available control samples for calibration purposes is a very simple and robust means for obtaining reliable results. Because the standards are treated

the same way as the subject samples and are present in a similar matrix, matrix effects are minimized. Use of primary standards in resource-limited settings is not recommended because of possible weighing and dilution errors; furthermore, different stability properties between primary standards and serum-based material can contribute to bias. Except for sTfR, all of the measured proteins have values assigned by Bio-Rad that can be used for calibration. Because the values differ slightly for the different methods, we used the mean value for our calibration curves. For sTfR we analyzed the Bio-Rad

**TABLE 3**

*Comparison of sandwich ELISA assay and traditional methods for serum ferritin, sTfR, RBP, and CRP*

	$n^1$	Assay	Mean	SD	$P^2$	Cutoff	$n^3$	$n^4$
Ferritin, $\mu$ g/L	41	Bio-Rad RIA	96.3	66.3	0.83	20	10	
		Sandwich ELISA	99.6	70.6				9
sTfR, mg/L	119	Ramco ELISA	10.8	4.9	0.93	8.3	79	
		Sandwich ELISA	10.7	3.9				85
RBP, $\mu$ mol/L	24	HPLC-UV	0.84	0.24	0.36	0.7	8	
		Sandwich ELISA	0.91	0.23				6
CRP, mg/L	17	IBL-ELISA	16.4	17.4	0.11	10	9	
		Sandwich ELISA	15.2	17.0				9

<sup>1</sup>  $n$  = number of subjects in sample set.

<sup>2</sup>  $P$  = paired  $t$  test to test the difference between samples measured by the traditional method and the sandwich ELISA.

<sup>3</sup>  $n$  = number of subjects classified as deficient based on cutoff value.

<sup>4</sup>  $n$  = number of subjects classified identically as deficient by the sandwich ELISA and the corresponding traditional method.

Liquicheck control using the Ramco assay because it is currently the most frequently used method for sTfR measurement. At present, sTfR kits from various manufacturers give different values for the same sample so the cutoff values for ID are kit-specific and must be adjusted.

Most ELISA reader software offers several different functions to calculate the standard curve (linear, semilog, log-log, quadratic, 4-parameter, cubic spline, exponential, log-logit, point-to-point). Because the range of our calibration curves covers the most important part of the physiological range, we chose to use the function with the best fit (best regression coefficient). In our experiments, a quadratic approximation usually had the best fit but a linear regression gave similar results.

Because the Bio-Rad Liquicheck controls were used for calibration, it was necessary to prepare our own high- and low-concentration QC serum pools by blending serum samples. These QC serum samples were divided into aliquots and kept frozen to be included with the standard calibration curve and subject samples during each assay. The QC serum pool target values and SD were established by performing multiple assays with the calibrators. Because Bio-Rad has control samples in the low and medium range they can also be used for this purpose, as could any other control material from other companies. In this regard, it is important to use HPLC-certified control materials for the RBP ELISA because the assay is used as a surrogate for serum retinol.

Although there are several methods available for the measurement of ferritin and the cost of the chemicals per measurement can be less than \$5, fewer methods are available for sTfR and the cost per measurement is approximately twice that of ferritin. To our knowledge this is the first paper describing the use of a sandwich ELISA technique with commercially available antibodies for sTfR. We selected antibodies from Hytest because the detection antibody is already conjugated with a peroxidase (similar to the Dako antibodies).

Retinol in serum measured by HPLC is the most widely used and accepted indicator for VA status (5). Because it is homeostatically regulated, it is not a good indicator when there are adequate liver stores of VA. Additionally, infection decreases the content of retinol in blood and complicates the interpretation of VA levels. Because retinol correlates well with RBP levels, RBP measurements can be used to estimate VA status. Under normal conditions, RBP in the blood is nearly saturated with retinol. At very low serum retinol levels ( $<0.35 \mu\text{mol/L}$ ) a portion of circulating RBP is not bound to retinol, resulting in a positive bias when reporting VA status (4). In comparison to HPLC, the RBP sandwich ELISA assay requires less technical expertise and provides higher throughput; however, its variability is higher than that of HPLC measurements. Because immunological techniques are known to be less accurate and less precise than chemical methods, we still recommend that HPLC measurements be performed

whenever possible. Another difficulty with the RBP sandwich ELISA assay, as well as the other ELISA methods, could be the availability of the peroxidase conjugated detection antibodies, i.e., if companies discontinue the production of these antibodies. With some technical experience, alternatives would be to prepare a conjugated antibody or to use a competitive ELISA.

An additional feature of the presented method is the provision of information on infectious status through the measurement of CRP. This was included to assist in the correction of VAD and ID prevalence rate. However, it is useful independently to measure the prevalence of acute infections in nutritional surveys. The CRP measurement can be easily integrated into our assay without adding much expense or work. For correcting values it would also be useful to measure  $\alpha 1$  acid glycoprotein as an indicator for chronic infections (7). The development and validation of this assay with the sandwich ELISA technique is currently underway.

We hope that the advantages described for these sandwich ELISA assays to measure iron and vitamin A status indicators plus indicators of infection and inflammation in  $30 \mu\text{L}$  of serum will increase the use of these methods to assess 2 of the most important nutritional deficiencies.

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