Validating a custom multiplex ELISA against individual commercial immunoassays using clinical samples

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The measurement of multiple antigens in a single sample poses clinical and methodological challenges. Here we describe the validation of a multiplexed sandwich enzyme-linked immunosorbent assay (ELISA) array (microELISA) of nine antigens. The antigens tested simultaneously were: α-fetoprotein (AFP), prostate specific antigen (PSA), carcinoembryonic antigen (CEA), cancer antigen 125 (CA 125), CA 15-3, CA 19-9, β-human chorionic gonadotropin (β-hCG), luteinizing hormone (LH), and follicle stimulating hormone (FSH). At least 44 clinical samples were tested for each antigen. microELISA results for the nine antigens were then compared with clinical laboratory results obtained for the same antigens in individual chemiluminescent immunoassays. The microELISA had a coefficient of variation (CV) of 7.3% within an assay and 12.6% for assays run at different times. A statistical comparison of results from the microELISA with results from the clinical laboratory showed that the assays had correlation coefficients ranging from 0.99 to 0.76, and Deming regression demonstrated that four of the nine assays were high-quality assays and not statistically different to the individual assays. To determine if the differences in the assays were due to methodology, the microELISA was also compared with conventional ELISAs using identical antibodies and reagents. Deming regression demonstrated that five of the eight assays were high-quality, indicating that a poor correlation between a microELISA and an individual immunoassay are partly due to antibody differences.

INTRODUCTION

Identifying multiple proteins in a single sample has advantages. In cancer diagnostics, specificity can be improved with more than one marker (1,2). For example, cancer antigen 125 (CA 125) is elevated in different types of cancer such as endometrial cancer (3), renal cell carcinoma (4), and ovarian cancer (5). In addition, if different diseases have overlapping clinical symptoms, the qualitative and quantitative assessment of multiple antigens may provide diagnosis. So, it has been reported that the same CA 125 antigen is also elevated in noncancerous conditions, such as endometriosis (6), mitral valve stenosis (7), and hypothyroidism (8,9). Therefore, if other markers can be identified to categorize the diseases, the potential benefits of measuring multiple antigens simultaneously from the same sample are obvious. Currently, technology is available for measuring multiple antigens, but the clinical interpretation is not available.

There are a variety of different methods available for identifying multiple antigens in the same sample simultaneously. The most common method for measuring multiple antigens from a complex mixture of proteins has been by two-dimensional gel electrophoresis (2-DE) (10,11), sometimes followed by identification by tandem mass spectrometry. Also available are protein arrays that require antibodies of known specificity and affinity (10,12) that are immobilized on a surface. Time-of-flight mass spectrometer techniques like matrix-assisted laser desorption/ionization (MALDI) and surface-enhanced laser desorption/ionization (SELDI) have also been used for identifying multiple antigens in complex mixtures (13,14).

Many methods allow parallel protein identification, but measurement of their concentrations is an important indicator used in both life science research and clinical practice (15). Arrays of antibodies for simultaneous antigen quantification are considered the most accurate (10,12). Enzyme-linked immunosorbent assay (ELISA) microarrays were first reported as printed arrays on glass (16). These arrays had 144 spots each that corresponded to the location of a well in a microtiter plate. A 16-spot array printed onto nitrocellulose attached to a microscope slide has also been reported (17). Microarrays can now be printed directly onto the bottom of a 96-well plate and have been used by different investigators and companies (15,18). For example, SearchLight™ arrays (Pierce Biotechnology, Rockford, IL, USA) have up to 16 assays per well in a sandwich format similar to our multiplexed sandwich ELISA array (microELISA) (15). When multicytokine assays based upon microELISA technology (SearchLight and FAST® Quant systems; Whatman Schleicher & Schuell, Keene, NH, USA) were compared with a bead-based array (Beadlyte® Luminex human multicytokine detection system; Upstate, Charlottesville, VA, USA) (19), each assay system had its own merits. For example, the FAST Quant assay tested had the broadest dynamic range and lowest sample volume requirements compared with the other assays, and the SearchLight assay had the best reported detection limits.

A rigorous comparison of microELISAs to identical assays performed as individual conventional ELISAs has not been performed. Our microELISA uses up to 25 antibodies bound to the bottom of a microtiter plate well in an array format. The microELISA was compared with routine clinical testing and identical conventional ELISAs using six tumor markers [α-fetoprotein (AFP), prostate specific antigen (PSA), carcinoembryonic antigen (CEA), CA 125, CA 15-3, and CA 19-9] and three hormones [β-human chorionic gonadotropin (β-hCG), luteinizing hormone (LH), and follicle stimulating hormone (FSH)].

MATERIALS AND METHODS

Samples

All samples were de-identified according to a global Associated Regional and University Pathologists (ARUP) Laboratories protocol under International Review Board (IRB) no. 7275. The samples used in this study had been submitted to ARUP for the quantification of AFP, total PSA, CEA, CA 125, CA 15-3, CA 19-9, β-hCG, LH, or FSH antigen levels. AFP, total PSA, CA 15-3, and CA 125 were measured on the Modular E170 analyzer (Roche Diagnostics, Indianapolis, IN, USA). CEA, CA 19-9, and β-hCG were measured on the Immulite® 2000 (Diagnostic Products Corporation, Los Angeles, CA, USA). LH and FSH were measured on the ADVIA Centaur® Immunoassay system (Bayer Healthcare, Tarrytown, NY, USA). Samples were either human serum or plasma (heparin or EDTA). Three hundred and sixty-four samples were tested by ARUP for one or two of the nine antigens. The only antigens that were measured on the same sample were LH and FSH; all other antigens were measured separately on different samples. Forty-four samples were tested for both FSH and LH, with three of those having LH results below the detection limit of the assay. Forty-six samples were tested for CA 125, 61 samples for CA 19-9, 19 samples for β-hCG, 55 samples for CEA, 37 samples for CA 15-3, 50 samples for PSA, and 52 samples for AFP. Upon completion of testing, the samples were stored at -80°C until transfer to the Spendlove Research Foundation (Logan, UT, USA) where the microELISA analysis was performed.

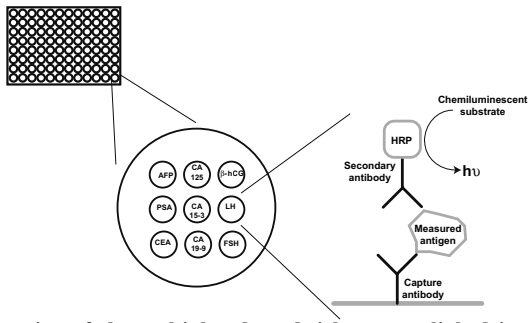

Figure 1. Representation of the multiplexed sandwich enzyme-linked immunosorbent assay (ELISA) array (microELISA) format and method. Nine antibodies specific for different antigens were spotted onto the bottom of a single well of a 96-well microtiter tray for simultaneous detection by a microELISA. AFP, α-fetoprotein; PSA, prostate specific antigen; CEA, carcinoembryonic antigen; CA 125, cancer antigen 125; CA 15-3, cancer antigen 15-3; CA 19-9, cancer antigen 19-9; β-hCG, β-human chorionic gonadotropin; LH, luteinizing hormone; FSH, follicle stimulating hormone; HRP, horseradish peroxidase.

Table 1. Supplier and Concentration of Each Antibody Used in the microELISA

	Antigen	Supplier	Catalog No.	Concentration (µg/mL)
Capture	AFP	OEM Concepts	M2-M01	50
Antibody	Total PSA	Fitzgerald	10-P20	100
	CEA	Fitzgerald	10-C10	40
	CA 125	BioDesign	M37197M	100
	CA 15-3	BioDesign	M37552M	160
	CA 19-9	BiosPacific	A46151130P	100
	β-hCG	Fitzgerald	10-C25	50
	LH	OEM Concepts	M2-F07	100
	FSH	OEM Concepts	M2-F02	100
Detection	AFP	Fitzgerald	10-A05	200
Antibody	Total PSA	BiosPacific	A45100151P	8
	CEA	OEM Concepts	M2-M02	1.6
	CA 125	BioDesign	M86306M	0.6
	CA 15-3	BioDesign	M37901M	40
	CA 19-9	Fitzgerald	10-C04	8.6
	β-hCG	Fitzgerald	10-C25	4
	LH	OEM Concepts	M2-F07	26.5
	FSH	OEM Concepts	M2-F02	1.2

microELISA, multiplexed sandwich enzyme-linked immunosorbent assay (ELISA) array; AFP, α-fetoprotein; PSA, prostate specific antigen; CEA, carcinoembryonic antigen; CA 125, cancer antigen 125; CA 15-3, cancer antigen 15-3; CA 19-9, cancer antigen 19-9; β-hCG, β-human chorionic gonadotropin; LH, luteinizing hormone; FSH, follicle stimulating hormone.

microELISA Analysis

Following testing at ARUP, the samples were shipped overnight in dry ice, stored at -20°C, and used within 1 year. Samples were never subjected to more than three freeze-thaw cycles. The nine microELISA assays were developed using commercially available antibodies that were diluted in phosphate-buffered saline (PBS), pH 7.4, and used at the concentrations given in Table 1. Antibodies and standardized antigens used in the microELISA were different than those used in the commercially available assays. Nine 50-nL spots of capture antibody were arranged in a 3×3 grid in each well of a 96-well plate (Greiner Bio-One, Monroe, NC, USA) (Figure 1) at 80% relative humidity using a PixSys™ Cartesian Dispensing system (Cartesian Technologies, Irvine, CA, USA). The plates were incubated for 4 h at 37°C (80% relative humidity) and then blocked in 100 µL/well Super AAA buffer (ScyTek, Logan, UT, USA) for 10 min at 20°C with shaking on an orbital shaker. All incubations to follow were performed similarly with shaking. After removal of the blocking solution, 30 µL sample or a 6-point standard curve (Lyphochek® Tumor Marker Control;

Table 2. Range and Sensitivity Specifications for the Nine Antigens Measured

	Clinical Assay		microELISA	
Antigen	**Range**	**Sensitivity**	**Range**	**Sensitivity**
AFP	0.6–1200 ng/mL	0.6 ng/mL	0.53–1300 ng/mL	0.53 ng/mL
Total PSA	0.01–100 ng/mL	0.003 ng/mL	1–48 ng/mL	0.04 ng/mL
CEA	0.2–560 ng/mL	0.2 ng/mL	0.02–510 ng/mL	0.02 ng/mL
CA 125	0.6–5000 U/mL	0.6 U/mL	0.88–5300 U/mL	0.88 U/mL
CA 15-3	1–300 U/mL	1 U/mL	0.28–310 U/mL	0.28 U/mL
CA 19-9	3–1000 U/mL	1 U/mL	0.07–240 U/mL	0.07 U/mL
β-hCG	1.1–5000 mIU/mL	0.6 mIU/mL	0.11–6300 mIU/mL	0.11 mIU/mL
LH	0.1–200 mIU/mL	0.1 mIU/mL	0.16–50 ng/mL	0.16 ng/mL
FSH	0.3–200 mIU/mL	0.3 mIU/mL	0.24–50 ng/mL	0.24 ng/mL

microELISA, multiplexed sandwich enzyme-linked immunosorbent assay (ELISA) array; AFP, α-fetoprotein; PSA, prostate specific antigen; CEA, carcinoembryonic antigen; CA 125, cancer antigen 125; CA 15-3, cancer antigen 15-3; CA 19-9, cancer antigen 19-9; β-hCG, β-human chorionic gonadotropin; LH, luteinizing hormone; FSH, follicle stimulating hormone; mIU, million international units.

Bio-Rad Laboratories, Hercules, CA, USA) diluted 1:10 in PBS, pH 7.4, containing 1% (w/v) bovine serum albumin (BSA) were added to the appropriate wells for 60 min at 20°C. Then the plates were washed three times in an Elx405™ microplate washer (BioTek Instruments, Winooski, VT, USA) with Tris-buffered saline (TBS), pH 7.4, containing Tween® 20 (ScyTek). Bound antigen was detected by adding 30 µL mixture of detection antibodies conjugated to horseradish peroxidase (HRP) (20) to the plate for 15 min at 20°C. Plates were washed three times as before, and 40 µL SuperSignal® ELISA Femto Chemiluminescent Substrate (Pierce Biotechnology) were added to produce a chemiluminescent response. The plate was imaged for 4 min using a Fluorchem® 8900 charge-coupled device (CCD; Alpha Innotech, San Leandro, CA, USA) with custom software. The software calculated the pixel intensity for each spot. Values were output to a spreadsheet for calculation of marker concentration using linear regression of the calibration curve. Each sample was tested in triplicate, and the average compared with standard curves to determine concentrations.

Conventional ELISAs

The same antibodies used in the microELISA format were also tested in a conventional ELISA format. This was to determine if there was any difference in results between the microELISA format and ELISAs that had been set up under conventional conditions. Eight different conventional ELISAs were performed. Each conventional ELISA had a separate random sample set ranging from 50 to 96 samples. The microELISA assays were performed at the same time as the conventional ELISAs on the same sample set to ensure similar reaction conditions. The conventional ELISA was performed identically to the microELISA except that antibodies were coated at a concentration of 2 µg/mL in PBS, pH 7.4, and the substrate incubation times ranged from 1 min to 1 h.

Precision Studies and Statistical Analysis

The array was tested for reproducibility over the course of 30 days by the same technician using different lots of reagents and plates. Twenty-four different 96-well plates were run with serum samples in triplicate, and the calibration curve in duplicate. Interassay precision was determined as the coefficient of variation (CV) of test samples at different dilutions over a period of 30 days (n = 24). Intra-assay precision was calculated by determining the CV over 96 different wells on the same plate. The test samples were diluted to be within the dynamic range of the assay calibration curve. Testing ranges were determined by regression statistics obtained from a comparison of methods study used to calculate the reference limits of a new method (21). The lower limits of detection (LLD) for each assay were calculated by adding the mean of the blank to two times the standard deviation of the blank.

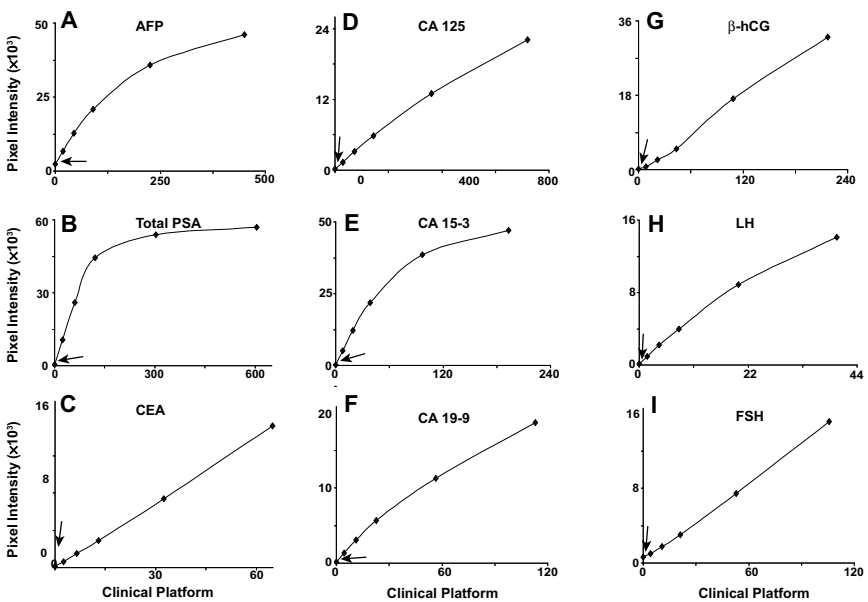

Figure 2. Standard curves for the nine antigens tested simultaneously in the multiplexed sandwich enzyme-linked immunosorbent assay (ELISA) array (microELISA). Each antigen is represented on a separate graph (A–I). The arrows indicate the lower limit of detection of each assay. AFP, α-fetoprotein; PSA, prostate specific antigen; CEA, carcinoembryonic antigen; CA 125, cancer antigen 125; CA 15-3, cancer antigen 15-3; CA 19-9, cancer antigen 19-9; β-hCG, β-human chorionic gonadotropin; LH, luteinizing hormone; FSH, follicle stimulating hormone.

microELISA array results were correlated to the reference methods and conventional ELISAs using Deming regression, which takes into account the uncertainty of both the reference and experimental assays. Statistical significance was determined by the 95% confidence intervals for the slope and intercept. In addition, assignment of "high quality" to an assay required that the intervals included a slope of 1 and an intercept of 0. Deming regressions were determined by EP Evaluator online at www.dgrhoads2.com.

RESULTS AND DISCUSSION

Nine different ELISAs were developed and multiplexed in array format in each well of a 96-well plate. The antigens AFP, PSA, CEA, CA 125, CA 15-3, CA 19-9, β-hCG, LH, and FSH were analyzed simultaneously. Cross-reactivity tests of nearly 14,000 points were performed by using each capture antibody with all potential combinations of antigen and detection antibodies to ensure minimal cross-reactivity with nonspecific factors (data not shown). The only cross-reactivity detected was using the antibody for CA 19-9, which detects Sial Lewis A carbohydrate moieties. However, assaying these samples with and without the CA 19-9 antibody did not affect the values for the microELISA.

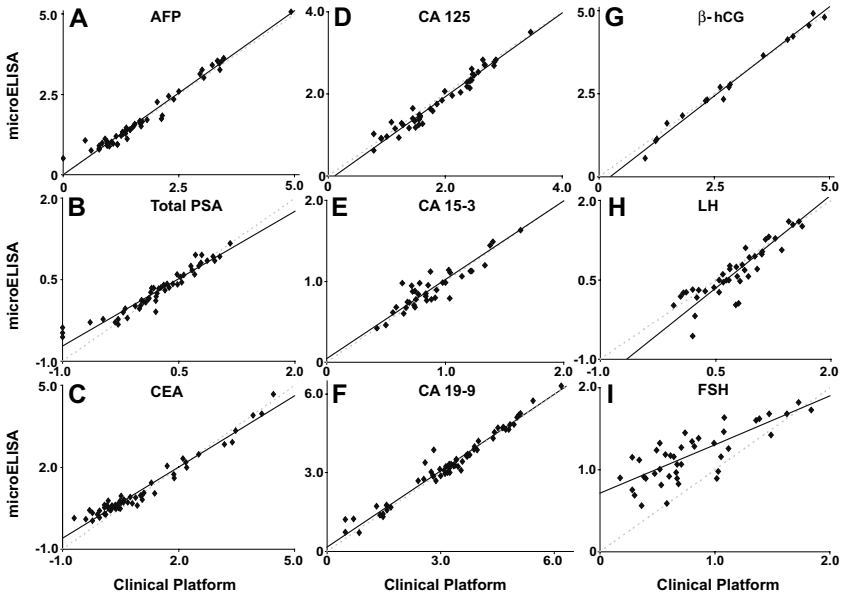

Figure 3. Regression plots of the results from each of the nine antigens measured on the multiplexed sandwich enzyme-linked immunosorbent assay (ELISA) array (microELISA) versus the corresponding results obtained from the individual clinical assays. Each antigen is shown on a separate graph (A–I). Each point represents a sample. The black line is the Deming regression and the gray dotted line represents a theoretical line with a slope of 1.0 and a y-intercept of 0. The logarithm of each result has been plotted. Units correspond to those of Table 2. AFP, α-fetoprotein; PSA, prostate specific antigen; CEA, carcinoembryonic antigen; CA 125, cancer antigen 125; CA 15-3, cancer antigen 15-3; CA 19-9, cancer antigen 19-9; β-hCG, β-human chorionic gonadotropin; LH, luteinizing hormone; FSH, follicle stimulating hormone.

The overall CV over 30 days was 12.63% with intra-assay variation contributing 7.26%. R^2 values were calculated for the standard curves of each antigen. Six-point standard curves, five serial dilutions of one to two, and a negative control were used, with the exception of PSA, where only three points were within the analytical measurement range (Figure 2). The R^2 values for the standard curves were 0.99 for FSH, CA 125, CA 19-9, β-hCG, CEA, and PSA; 0.98 for LH; 0.92 for AFP; and 0.88 for CA 15-3. The sensitivity and useful ranges of the microELISA were compared with conventional clinical assays (Table 2). The CA 15-3, CEA, β-hCG, and CA 19-9 assays were more sensitive by microELISA, while total PSA, CA 125, and LH assays were more sensitive by clinical laboratory testing. The β-hCG assay had a broader analytical measurement range by microELISA, while total PSA, CA 19-9, LH, and FSH had a broader analytical measurement range by clinical laboratory testing.

Deming regression analysis indicated that four of the nine microELISA assays (AFP, CA 125, CA 15-3, and CA 19-9) were not statistically different from the clinical assays (Table 3 and Figure 3). For the remaining antigens, either the slopes were different with a proportional difference between the methods, and/or the intercepts were different with a

Table 3. Deming Regression Data for the Nine Antigens Tested Simultaneously in the microELISA Versus the Individual Clinical Assays

	Slope	95% CI	Intercept	95% CI	SEM	R^2	*n*
AFP[a]	1.02	0.97 to 1.07	-0.01	-0.11 to 0.1	0.18	0.99	52
Total PSA	0.83	0.76 to 0.89	0.2	0.14 to 0.26	0.16	0.96	50
CEA	0.87	0.82 to 0.92	0.27	0.19 to 0.35	0.22	0.98	55
CA 125[a]	1.02	0.96 to 1.09	-0.12	-0.24 to 0.01	0.14	0.98	46
CA 15-3[a]	0.97	0.83 to 1.12	0.09	-0.18 to 0.37	0.23	0.9	37
CA 19-9[a]	0.97	0.92 to 1.02	0.17	-0.01 to 0.34	0.24	0.98	61
β-hCG	1.07	1.02 to 1.13	-0.25	-0.43 to -0.06	0.15	0.99	19
LH	1.17	0.98 to 1.35	-0.24	-0.4 to -0.08	0.25	0.88	41
FSH	0.59	0.45 to 0.73	0.72	0.59 to 0.84	0.21	0.76	44

Data shown are the slope and Y-intercepts with their 95% confidence intervals (CI), standard error (SE), correlation coefficients (R^2), and the number of samples used in the calculations (*n*). microELISA, multiplexed sandwich enzyme-linked immunosorbent assay (ELISA) array; AFP, α-fetoprotein; PSA, prostate specific antigen; CEA, carcinoembryonic antigen; CA 125, cancer antigen 125; CA 15-3, cancer antigen 15-3; CA 19-9, cancer antigen 19-9; β-hCG, β-human chorionic gonadotropin; LH, luteinizing hormone; FSH, follicle stimulating hormone.
[a]Indicates high-quality assays with no significant difference to clinical assays because CI included slopes of 1.0 and intercepts at 0.0.

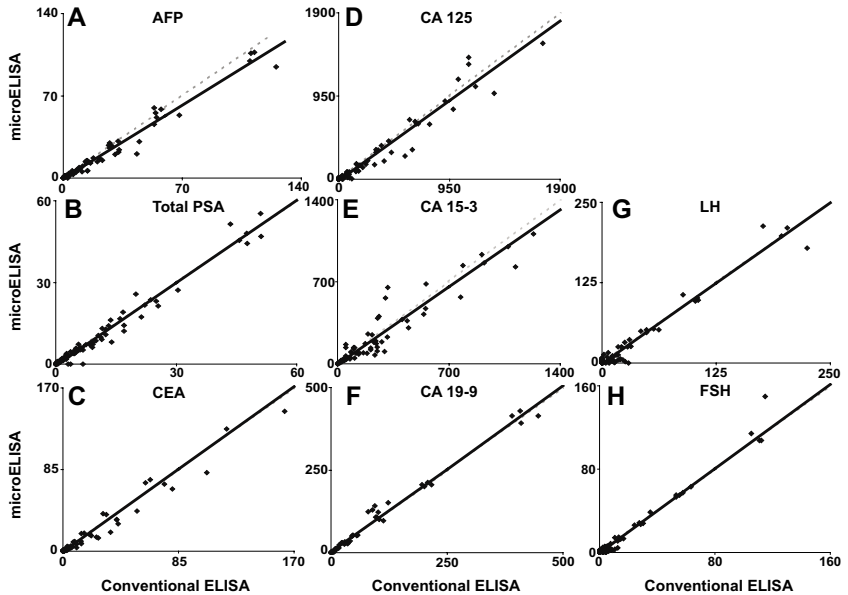

Figure 4. Regression plots of the results from each of the eight antigens measured on the multiplexed sandwich enzyme-linked immunosorbent assay (ELISA) array (microELISA) versus the corresponding results obtained from the conventional ELISAs using identical antibodies. Each antigen is shown on a separate graph (A–H). Each point represents a sample. The black line is the Deming regression and the gray dotted line represents a theoretical line with a slope of 1.0 and a Y-intercept of 0. Units correspond to those of Table 2. AFP, α-fetoprotein; PSA, prostate specific antigen; CEA, carcinoembryonic antigen; CA 125, cancer antigen 125; CA 15-3, cancer antigen 15-3; CA 19-9, cancer antigen 19-9; LH, luteinizing hormone; FSH, follicle stimulating hormone.

constant offset between assays (22,23). The PSA, CEA, β-hCG, and FSH had both a proportional and constant difference between assays. For LH there was a constant offset between the clinical assay and the microELISA.

One of the challenges in antibody microarray development is to match the specificity of antibodies used in conventional assays. For example, posttranslational modifications of FSH may differ between patients, and the antibodies recognizing FSH in the microELISA and the conventional assay may recognize different epitopes (24,25). To test this hypothesis, conventional ELISAs were performed using identical antibodies as those in the microELISA to confirm that the differences seen compared with the commercial assays were due to different antibodies. Five out of the eight assays were statistically identical according to the Deming regressions (Figure 4 and Table 4). This suggests that different antibodies are at least partly responsible for differences between the microELISA and the clinical platforms. Antibody variation is a general problem for all arrays using an immunoassay approach, and the development of databases of antibody reactivity will improve comparisons (26).

High-quality microELISA assays can be developed with characteristics similar to macroassays run on clinical platforms. Further improvements in antibody selection are expected to improve correlations with existing assays. microELISA arrays have promise for clinical diagnostics, especially when the available sample is limited. With specific antigen or biochemical panels, diagnosis and prognosis may increase in accuracy, leading to lower patient morbidity and mortality.

Table 4. Deming Regression Data for the Eight Antigens Tested Simultaneously in the microELISA Versus Conventional ELISAs Using Identical Antibodies

	Slope	95% CI	Intercept	95% CI	SE	R^2	*n*
AFP	0.9	0.86 to 0.93	-0.54	-1.86 to 0.78	3.41	0.97	73
Total PSA[a]	1.01	0.98 to 1.04	-0.49	-0.99 to 0.01	1.4	0.97	96
CEA	0.93	0.89 to 0.97	-0.46	-1.59 to 0.68	3.5	0.97	89
CA 125[a]	0.96	0.91 to 1.01	-15.97	-37.11 to 5.18	59.62	0.94	88
CA 15-3[a]	0.94	0.88 to 1.00	-3.93	-22.87 to 15.02	55.55	0.91	96
CA 19-9[a]	1.01	0.97 to 1.04	2.49	-2.27 to 7.74	10.11	0.99	50
LH[a]	1.0	0.96 to 1.04	-0.89	-2.95 to 1.17	6.51	0.96	96
FSH	1.08	1.04 to 1.11	-0.81	-1.78 to 0.17	2.87	0.98	92

Data shown are the slope and Y-intercepts with their 95% confidence intervals (CI), standard error (SE), correlation coefficients (R^2) and the number of samples used in the calculations (*n*). microELISA, multiplexed sandwich enzyme-linked immunosorbent assay (ELISA) array; AFP, α-fetoprotein; PSA, prostate specific antigen; CEA, carcinoembryonic antigen; CA 125, cancer antigen 125; CA 15-3, cancer antigen 15-3; CA 19-9, cancer antigen 19-9; LH, luteinizing hormone; FSH, follicle stimulating hormone.
[a]Indicates high-quality assays with no significant difference to clinical assays because CI included slopes of 1.0 and intercepts at 0.0.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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