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Outcome Validation of the Beckman Coulter Access Analyzer in a Second-Trimester Down Syndrome Serum Screening Application

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Background: **Mid-trimester maternal serum α-fetoprotein (AFP) and unconjugated estriol (uE3) are 30% lower and human chorionic gonadotropin (hCG) is twofold higher in Down syndrome pregnancies compared with unaffected pregnancies. In maternal serum screening, patient-specific risks are calculated using published gaussian frequency distribution parameters for these three markers obtained with previously available immunoassays. New immunoassays must generate similar distribution parameters if the accuracy of assigned risks and overall performance of prenatal screening are to be maintained.**

Methods: **Agreement between the Beckman Coulter Access and the Bayer Immuno 1 assays for AFP and hCG and the Amersham Amerlex-M RIA for uE3 was assessed in 558 fresh sera. Precision was measured over 6 weeks. Median concentrations were calculated by regression of 568 Caucasian singleton pregnancy samples against gestational age in days. Frozen mid-trimester sera from 44 confirmed Down syndrome singleton pregnancies (cases) were selected without conscious bias for reanalysis, and each case was matched with five control specimens from unaffected pregnancies. Serum markers were expressed as the multiple of the median (MoM) concentration derived from the control samples, corrected for maternal weight and converted to their log-equivalent values. Normality was assessed using probability plots and the Shapiro–Wilk *W*-test. Gaussian distribution parameters were compared with established values, and Down syndrome risk calculations were assessed with a commonly used risk algorithm.**

Results: **The Access AFP and hCG assays had consistent proportional agreement with the established assays, whereas agreement between the uE3 methods was less consistent. Analytical imprecision was 3–6% at mid-trimester concentrations. Normal distributions were obtained for the log MoM values of all three markers in both the Down syndrome and unaffected populations, and their gaussian distribution parameters compared well with established values. The performance of the Access assays in an established trivariate risk algorithm for Down syndrome was equal to the performance exhibited by traditional methods.**

Conclusion: **The Beckman Coulter Access analyzer provides valid mid-trimester serum AFP, uE3, and hCG results and risk assessments when applied in a prenatal Down syndrome screening service.**

Prenatal screening for fetal trisomies in the second trimester of pregnancy based on maternal serum markers commonly relies on three assays, the triple marker screen of α-fetoprotein (AFP),[1] unconjugated estriol (uE3), and chorionic gonadotropin (hCG). The measurement of these three normally occurring constituents in second-trimester maternal serum enables the assignment of a patient-specific risk of fetal trisomy 21 (Down syndrome) *(1)*. In North America, serum "triple marker screening" super-

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[1] Nonstandard abbreviations: AFP, α-fetoprotein; uE3, unconjugated estriol; hCG, human chorionic gonadotropin; LMP, last menstrual period; U/S, ultrasound; and MoM, multiple(s) of the median.

seded single-marker AFP screening in the early 1990s *(2)*, whereas in Europe the addition of the estriol marker was somewhat slower but is now more widely used. Maternal serum uE3 improves the detection rate for Down syndrome by 5–10% *(3)* while reducing the screen-positive rate sufficiently to justify the cost of its measurement *(4)*. In addition, uE3 is the best single biochemical marker for fetal trisomy 18 or Edwards syndrome *(5)*. A recently published screening algorithm for Smith–Lemli–Opitz syndrome (a disorder of cholesterol biosynthesis) relies on the finding of an isolated low uE3 concentration in the presence of AFP and hCG concentrations close to their respective median values *(6)*.

Assays used in a screening program require long-term stability because the results of previously screened women serve as the reference for those currently being screened. Once established, analyzers and analytical systems used in screening are not frequently changed, partly because of the arduous task to validate the performance of a new assay in detecting the targeted disorder, Down syndrome, which occurs only once in 600 pregnancies. Validation of an assay is best achieved in an unbiased population with complete ascertainment of Down syndrome pregnancy outcomes *(7)*.

Automated immunoassay systems for AFP and hCG have existed for many years. However, until recently the measurement of uE3 has relied on manual or semi-automated immunoassays. The Amerlex-M Free Estriol Second Trimester RIA (Amersham) is traceable to the first published studies of uE3 in Down syndrome screening *(8)*, but this assay was recently discontinued. Another RIA (DSL) for uE3 is now the most commonly used in North America and, in combination with AFP and hCG, has been shown to generate accurate triple marker risks of Down syndrome *(9)*. Several automated immunoassays for uE3 have been marketed in the past 5 years, all designed primarily for third-trimester fetal assessment. Despite this, the assays are sensitive enough to enable measurement of the second-trimester uE3 concentrations necessary for prenatal screening.

Maternal serum uE3 concentrations are, on average, 25–30% lower in fetal Down syndrome pregnancies compared with unaffected pregnancies *(3)*. However, varying agreement between two uE3 assays could potentially diminish the observed separation between the Down syndrome and unaffected populations and reduce the effectiveness of uE3 in prenatal screening. There are ~3 million women screened annually in North America, many with the new automated uE3 assays. To date there have been no published reports that validate the use of the new uE3 assays in a Down syndrome screening application. Part of the present study was previously presented at the American Association for Clinical Chemistry annual meeting in 2000 *(10)*.

Materials and Methods

AGREEMENT WITH EXISTING METHODS

The study was performed in the setting of a large multisite second-trimester maternal serum prenatal screening service. The assays in use at the time of the study were the Bayer Immuno 1 (Bayer Diagnostics) for AFP and hCG and the Amerlex-M Free Estriol Second Trimester RIA (Amersham IM 4041; Ortho-Clinical Diagnostics) for uE3. In the study, Beckman Coulter Access reagents for AFP, uE3, and hCG (cat. nos. 33210/33211, 33570, and 33500 with their corresponding calibrator products) were used on an Access I analyzer. The agreement between the Access and the currently used assays was assessed over a 6-week period in 558 patients with gestational ages between 14 and 22 weeks. All specimens were centrifuged and transported to the reference laboratory (usually within 2 days) where they were refrigerated until assayed the next working day. Agreement was assessed using a Bland–Altman percentage difference plot expressing the difference between the methods as a percentage of the mean value observed with the two methods *(11)*.

PRECISION OF THE ACCESS METHODS

During the agreement study, the precision of the Access assays was estimated using the three-level Bio-Rad Lyphochek Maternal Serum Control sera (cat. no. 2510; Bio-Rad Laboratories). Three levels of mid-trimester pooled sera (aliquoted and frozen) were included in the precision assessment of uE3.

OUTCOME VALIDATION STUDY SPECIMEN SELECTION

A case-control design study was used to examine the performance of the Access assays in a Down syndrome screening application. For comparison with the Access uE3 assay, the Amerlex-M RIA was used because its antibody source can be traced to the first published prenatal screening studies *(8)*. For AFP and hCG, the comparator assays were performed on the Bayer Immuno 1 analyzer. Second-trimester serum specimens from 44 singleton Down syndrome Caucasian pregnancies were retrieved from storage (−20 to −70 °C). The specimens were selected without conscious bias from a larger serum bank of 145 Down syndrome-affected pregnancies that had been identified with essentially complete pregnancy outcome ascertainment *(12)*. All stored specimens were from a screened population of 91 000 women who chose to have prenatal serum screening between 1993 and 1995.

Samples were identified by a coded number, and Research Ethics Board (Institutional Review Board) approval for the study was obtained. Each Down syndrome "case" specimen was matched with 5 control specimens (220 control specimens in total) from singleton unaffected pregnancies as follows: specimen storage environment (identical); gestational age (usually identical, ± 3 days maximum); maternal age (usually ± 12 months); racial origin (identical, Caucasian); nondiabetic status (identical). Maternal weight and the method of assignment of

gestational age, either by last menstrual period (LMP) date or by ultrasound (U/S) biometry, were known for all specimens. The gestational age was assigned by LMP in 52% of the Down syndrome cases and in 40% of the unaffected controls. The maternal age distributions in both the Down syndrome and unaffected populations were normally distributed with means (SD) of 33.5 (5.3) years in the Down syndrome pregnancies and 33.3 (5.1) years in the controls. The mean maternal weight was 150 pounds in both the cases and the controls. Sample stability during storage was verified by measuring uE3 with the Amerlex-M RIA on the thawed 44 case and 199 control samples and comparing these with the original fresh-sample results from the same assay. Agreement was assessed using the Bland–Altman percentage difference plot *(11)*.

CALCULATION OF MULTIPLE OF THE MEDIAN RESULTS

The case-control samples were thawed and assayed for AFP, uE3, and hCG with the Access reagent systems; uE3 was also assayed with the Amerlex-M RIA. The original Amerlex-M uE3 assay fresh-specimen results when the patients were first screened were also retrieved. Two control samples were removed because their AFP concentrations when originally reported were below the 1st and above the 99th percentiles of the unaffected population. The concentrations of all three markers from all assay sources were expressed as the multiple of the unaffected median (MoM) values calculated by regression of all 218 control samples against their respective gestational ages. Each MoM value was corrected for maternal weight by the weighted log-linear method *(13)*. All weight-corrected MoM values were converted to their log equivalents. Log MoM values in the control samples were adjusted by subtracting the observed mean log value from each control sample to achieve an unaffected population mean log MoM value of zero; corresponding case samples were then adjusted with the same value.

ANALYSIS OF GAUSSIAN DISTRIBUTIONS

The log weight-corrected MoM values for each marker, each population, and each assay source were assessed for their fit to the gaussian distribution by use of a gaussian distribution plot followed by a Shapiro–Wilk analysis *(14)*. In one case sample, the original Amerlex-M uE3 result (0.15 MoM) was below the 1st percentile, and this sample was removed from the analysis of results of that method only. With this exception, the fit of the data to the expected gaussian distributions was such that the mean values and SDs were calculated from the entire populations without truncation. The Pearson correlation coefficient was calculated for each paired combination of the markers for all assays in both the Down syndrome and unaffected populations.

SCREENING PERFORMANCE ASSESSMENT

The risk of Down syndrome was calculated in the 44 Down syndrome cases and their respective control samples by use of the weight-corrected MoM results for AFP, uE3, and hCG from the Access analyzer. We used the 1988 risk algorithm of Wald et al. *(1)* with the updated gaussian distribution parameters from their 1994 publication *(15)*. For comparison, the test results from the original fresh-sample analyses were expressed as weight-corrected MoM values in the same manner as the case samples and used to calculate risks. The risk results from both analytical systems were then subjected to ROC analysis *(16)*.

MEDIAN STUDY

In the agreement study, 467 specimens were from Caucasian pregnancies with gestational ages between 15 and 20 weeks, predominantly 15–17 weeks. To obtain mid-trimester median concentrations for clinical use, 101 frozen sera (unrelated to the case-control specimens) from Caucasian patients in gestational weeks 18–20 were thawed and analyzed with the study assays. There was no evidence of deterioration in the thawed specimens, and their

Table 1. Precision of the Beckman Coulter Access uE3, AFP, and hCG assays at various mid-trimester concentrations.

	uE3, μg/L					
Week 16 MoM equivalent	**0.3**	**0.6**	**0.9**	**1.4**	**1.7**	**4.7**
Mean	0.27	0.55	0.86	1.27	1.52	4.30
SD	0.023	0.025	0.044	0.076	0.124	0.262
CV, %	8.3	4.4	5.2	6.0	8.1	6.1
n	24	23	23	21	24	22
Source	Bio-Rad	Pool	Pool	Pool	Bio-Rad	Bio-Rad
	AFP, μg/L			**hCG, kIU/L**		
Week 16 MoM equivalent	**0.4**	**0.9**	**2.2**	**0.4**	**1.1**	**2.1**
Mean	13.3	35.1	83.5	12.0	38.4	70.2
SD	0.42	1.06	3.46	0.73	1.30	3.24
CV, %	3.1	3.0	4.1	6.1	3.4	4.6
n	25	27	27	24	22	23

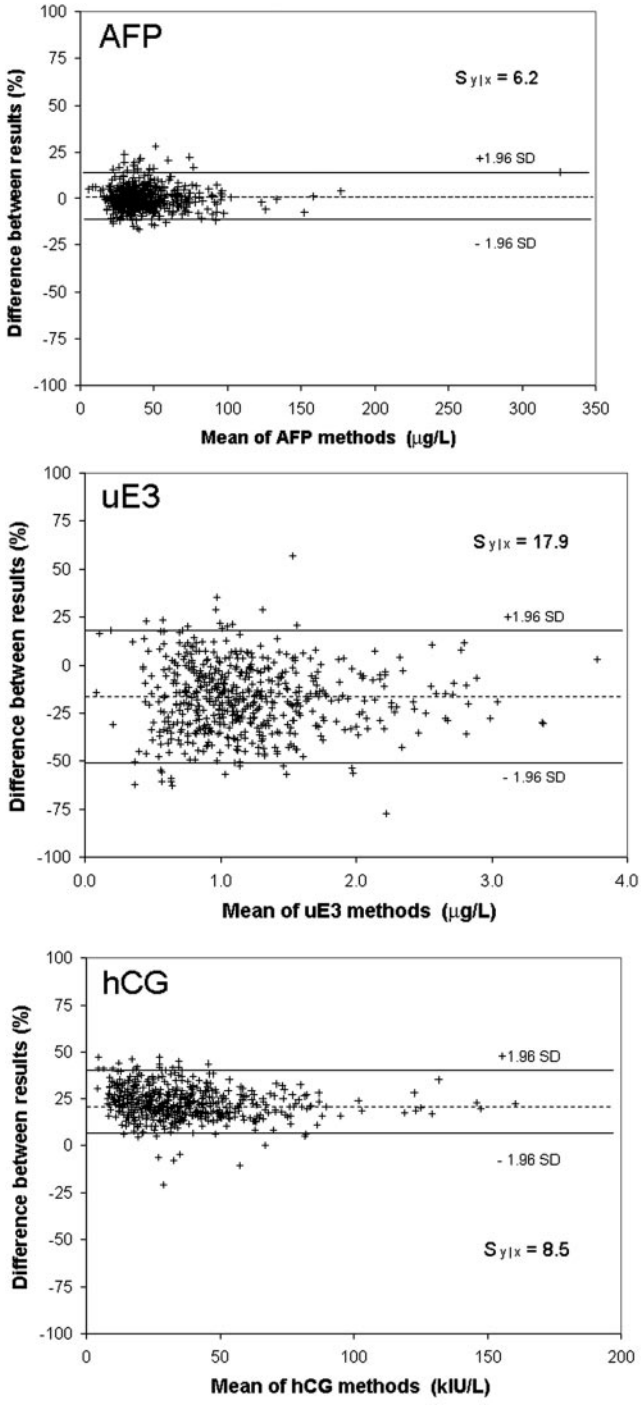

Fig. 1. Method agreement study.

Agreement between the Beckman Coulter Access and comparator methods for AFP (*top*), uE3 (*middle*), and hCG (*bottom*) in 558 fresh mid-trimester patient sera. The comparator method for AFP and hCG was the Bayer Immuno 1; for uE3, the comparator method was the Amerlex-M Mid-trimester RIA.

results were combined with those from the agreement study (568 in total, with 66% of gestational ages assigned by U/S biometry). Midweek median concentrations for weeks 15–20 were calculated with use of the log-linear curve fit for AFP and uE3 and the modified exponential curve fit for hCG *(17)*.

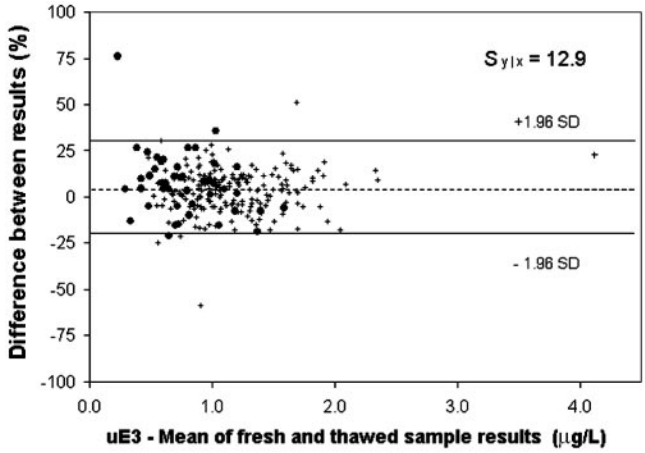

Fig. 2. Stored sample stability.

Agreement between the original fresh-specimen Amerlex-M uE3 assay results and the results with the same assay after several years of frozen storage for mid-trimester sera from 44 Down syndrome affected pregnancies (●) and 199 matched unaffected pregnancies (+).

Results

PRECISION

The precision of the Access AFP, uE3, and hCG assays for mid-trimester concentrations is presented in Table 1. The Bio-Rad quality-control material spans the range of both the second- and third-trimester uE3 concentrations. To obtain sufficient information in the range of concentrations of a prenatal screening application, pooled mid-trimester sera were also assayed for uE3 only, as shown in Table 1 where the mean concentrations are also expressed as the approximate MoM values for a gestational age of 16 weeks.

METHOD AGREEMENT IN FRESH SERA

The agreement between the Access assays and the methods in current use for the fresh maternal serum samples is shown in Fig. 1. The agreement between the Immuno 1 and Access methods for AFP was consistent within the range of concentrations encountered, and the SD of the percentage residuals was small (6.2%). For hCG, a constant proportional bias was detected, with the Access values 22% higher than the Immuno 1. The SD of the percentage residuals was 8.5%. The least agreement between methods was seen with uE3, where the Access yielded 17% lower values and the SD of the percentage residuals was 17.9%.

A proportional bias between assays can be eliminated by expressing all results in MoM values, the multiple of the median value in the unaffected population, but only if the bias is consistent. The percentage agreement between the methods for uE3 was typical of a steroid assay and would not be fully eliminated by expressing the results in MoM values.

STABILITY OF THE OUTCOME VALIDATION SAMPLES

The agreement (Fig. 2) between the original fresh-sample Amerlex-M uE3 results and the reassayed results with the

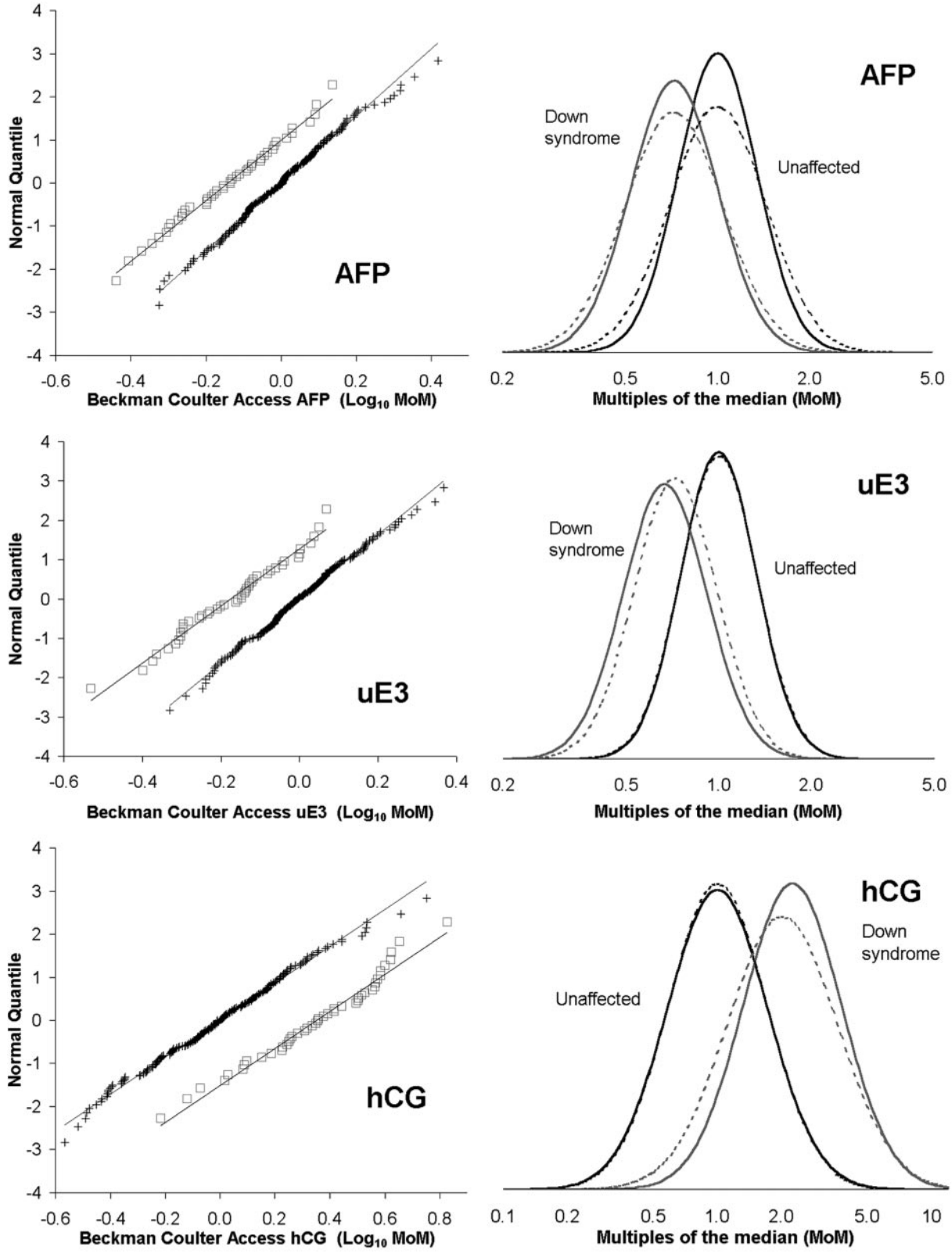

Fig. 3. Gaussian distributions.

Normality plots (*left*) and the resulting frequency distribution plots (*right*) of the observed unaffected (+) and Down syndrome (□) $\log_{10}$ MoM results obtained with the Access analyzer for AFP, uE3, and hCG after correction for maternal weight. In the normality plots, the fit of the data to a straight line indicates a gaussian distribution. For the frequency distribution plots, *solid lines* represent the Access results and *dashed lines* represent the results obtained by Wald et al. in 2000 *(18)* for AFP (*top*) and the results obtained by Wald et al. in 1994 *(15)* for uE3 (*middle*) and hCG (*bottom*).

Table 2. Gaussian frequency distribution variables.[a]

Population means and SDs

	uE3				AFP		hCG	
	Beckman Coulter	Wald 1994 *(15)*[b]	Original Amerlex-M[c]	Reassayed Amerlex-M[c]	Beckman Coulter	Wald 1994 *(15)*; Wald 2000 *(18)*[b]	Beckman Coulter	Wald 1994 *(15)*[b]
Down syndrome								
Log_{10} mean	−0.1749	−0.1411	−0.1786	−0.1760	−0.1417	−0.1427	0.3498	0.3023
Mean MoM	0.67	NA[d]	0.66	0.67	0.72	NA	2.24	NA
Median MoM	0.70	0.72	0.67	0.68	0.73	0.72	2.28	2.01
Log_{10} SD	0.1363	0.1336	0.1644	0.1559	0.1412	0.1595	0.2284	0.2563
Unaffected log_{10} SD	0.1220	0.1238	0.1226	0.1218	0.1281[e]	0.1559[e]	0.2333	0.2288

Correlation coefficients between log_{10} MoM marker pairs

	Down syndrome		Unaffected	
	Beckman Coulter	Wald 1994 *(15)*; Wald 2000 *(18)*[b]	Beckman Coulter	Wald 1994 *(15)*; Wald 2000 *(18)*[b]
AFP & uE3	0.2548	0.2523	0.1420	0.1664
AFP & hCG	0.0812	0.2394	0.0805	0.0644
uE3 & hCG	0.0425	−0.3690	−0.1524	−0.1137

[a] Shown are the means and SDs for the maternal weight-corrected log_{10} MoM values of AFP, uE3, and hCG obtained with the Beckman Coulter Access for mid-trimester sera from 44 Down syndrome pregnancies and 218 matched unaffected pregnancies, together with the correlation coefficients between the paired combinations of the markers and associated published reference values.

[b] Published data are for maternal weight-corrected populations with gestational age assigned 50% by LMP and 50% by U/S from Wald and coworkers *(15, 18)*.

[c] Original and reassayed Amerlex-M uE3 log_{10} MoM distributions are based on regressed medians obtained in the present study.

[d] NA, not applicable.

[e] Access log_{10} AFP SD significantly smaller than the corresponding reference value ($P < 0.001$).

same method indicated that the samples were stable during frozen storage. The bias between fresh and frozen samples was 0.04 μg/L and would not affect subsequent calculations of MoM values or Down syndrome risks. The SD of the percentage residuals in the Bland–Altman plot (12.9%) was significantly smaller ($P < 0.001$, *F*-test) when the same assay was used for both the fresh and stored samples, compared with the values shown in Fig. 1, where different uE3 methods were used in the fresh specimens.

ASSESSMENT OF GAUSSIAN DISTRIBUTIONS

Normality plots of the maternal weight-corrected log MoM results for the three markers in the Down syndrome and unaffected populations are displayed in Fig. 3, to-

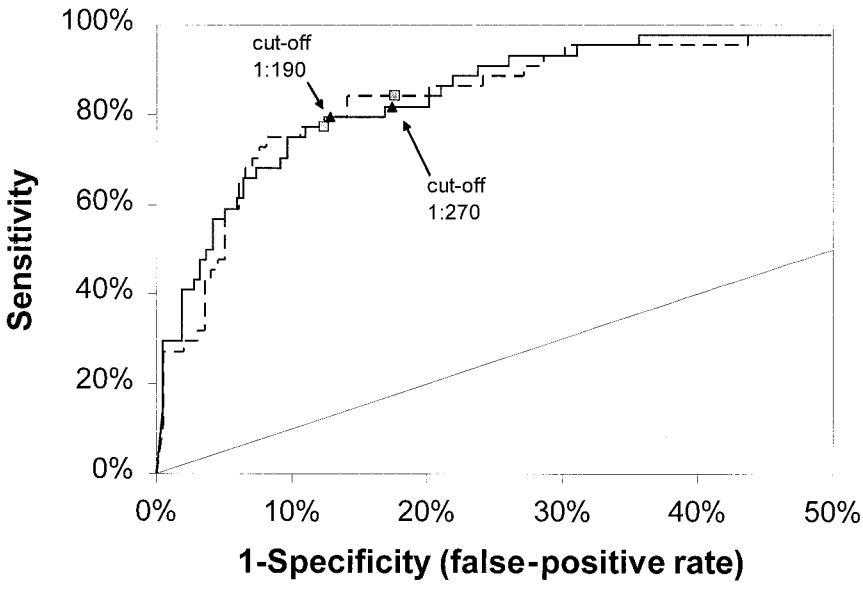

Fig. 4. Performance in Down syndrome screening.

ROC curve for the calculated Down syndrome risks in 44 Down syndrome mid-trimester specimens and 218 (age-matched) unaffected controls, using the results from the originally performed (fresh-specimen) assay (*dashed line*) and those derived from the Beckman Coulter Access AFP, uE3, and hCG assays (*solid line*) after specimen storage. Risks were calculated using the trivariate gaussian distribution algorithm *(1)* and the population parameters from Wald et al. *(15)*. Performance at two commonly used mid-trimester risk cutoffs is shown (*arrows*). The *diagonal line* indicates no discrimination. *Dashed line*, original methods: *solid line*, Access methods.

Table 3. Median values, with compartor medians for uE3.[a]

Gestational age weeks/days	n	uE3, μg/L			AFP, μg/L	hCG, kIU/L
		Access	Amerlex	Current[b]		
15/3	105	0.73	0.88	0.88	33.3	42.4
16/3	199	0.92	1.10	1.09	37.6	34.1
17/3	80	1.15	1.37	1.35	42.5	28.3
18/3	72	1.43	1.70	1.67	48.0	24.1
19/3	71	1.79	2.11	2.08	54.3	21.2
20/3	41	2.24	2.62	2.58	61.3	19.1

[a] Shown are regressed mid-trimester Caucasian median concentrations for AFP, uE3, and hCG on the Beckman Coulter Access analyzer, in a population with gestational ages assigned by U/S biometry in 66% of patients.

[b] Medians in current use were based on 6 months of previous experience with the Amerlex-M assay.

gether with the calculated gaussian frequency distributions. No samples were excluded, and in each case the Access data series adhered to the gaussian distribution as indicated by the Shapiro–Wilk *W* test (minimum observed *P* value, 0.420; low *P* values indicate that the population is nonnormally distributed). The observed values for themeans, SDs, and correlation coefficients, all of which are required for the calculation of risk of Down syndrome, are presented in Table 2.

PERFORMANCE IN DOWN SYNDROME SCREENING

The ROC plot of the fraction of Down syndrome cases that would be screen positive vs the false-positive rate over a range of risk cutoffs is shown in Fig. 4. The two commonly used mid-trimester risk cutoffs of 1:270 and 1:190 are identified in Fig. 4. The area under the ROC curve was highly significant ($P < 0.001$) for both the original methods (area, 0.90) and for the Access methods (area, 0.91).

MEDIAN STUDY

The regressed median values obtained using the Access AFP, uE3, and hCG assays for the 568 median study samples from Caucasian patients are presented in Table 3. The subpopulation base for these Access medians is reliable, as indicated by the fact that the Amerlex-M medians from the same patients were very similar to the medians in use for that assay from a much larger population. The Access uE3 medians increased at a rate of 25% per week from week 15 through week 20, and for AFP the rate of increase was 13%. Both of these are consistent with expected performance in a population with gestational age assigned by ultrasound in 66% of patients *(17)*. The medians for hCG decreased from week 15 through week 20, and the indicator ratio of the week 15 median to week 20 was 2.2.

Discussion

In a prenatal screening application, maternal serum marker concentrations are measured, expressed as the multiple of the unaffected population median concentration (MoM), and then used in a risk calculation based on population parameters for each marker derived from the assays used in the original publication.

Most risk-calculation software programs use the Down syndrome risk algorithm based on the trivariate gaussian distributions published by Wald et al. *(1)*. The original population parameters were subsequently updated in 1994 *(15)* and again in 2000 *(18)* for AFP. Two reliable studies have since validated the assigned risks against the observed prevalence of Down syndrome in screened populations *(9, 19)*. The present study compared the population parameters from the Access assays for AFP, uE3, and hCG against an appropriate subset of the 1994 and 2000 parameters *(15, 18)*, which are included in Table 2.

The use of the MoM unit is intended to reduce or eliminate significant differences between analytical systems and populations screened. However, the MoM unit is still susceptible to differences between assays in their specificity for the target marker, among other factors. If the MoM distributions of both the Down syndrome and unaffected populations with a new assay do not closely approximate the population parameters used in the subsequent risk calculation, the risks assigned to patients can be both over- and underestimated. Therefore, several criteria must be met in assessing the validity of AFP, uE3, and hCG assays for a Down syndrome screening application. The log MoM frequency distributions in both populations (unaffected and Down syndrome) should be gaussian because the likelihood ratio calculation used in assigning the risk of Down syndrome assumes this. The population means and SDs for each marker in both populations should be similar to those used in the risk calculation, and the same is true for the correlation coefficients between marker pairs in both populations *(3)*.

The results in Figs. 3 and 4 and in Table 2 demonstrate that the Access AFP, uE3, and hCG assays meet the criteria for use in a Down syndrome screening application. For uE3 and hCG, the separation between the means of the unaffected and Down syndrome populations is slightly greater than reported in the reference data; however, the reference means are within the 95% confidence interval of the Access means. The Access AFP data had smaller SDs than the reference data, significantly so in the unaffected population ($P < 0.01$). Improved analytical precision yields a lower population SD *(18)*. Other factors

that affect the population variance are the accuracy of gestational age assignment *(20)* and the extent to which maternal weight correction has been performed. The reference data were selected to mimic both the percentage of U/S and LMP dating for gestational age assignment and the complete weight correction in our study. Therefore, greater assay precision is likely responsible for the smaller unaffected population SD for AFP.

The Beckman Coulter assays for the three second-trimester maternal serum screening markers, AFP, uE3, and hCG, are suitable for assessments of Down syndrome risk using the trivariate gaussian distribution algorithm.

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