

**Comparison of Two Automated Adrenocorticotrophic Hormone Assays,** Michael Vogeser,<sup>1\*</sup> Dieter Engelhardt,<sup>2</sup> and Karl Jacob<sup>1</sup> (<sup>1</sup> Institute of Clinical Chemistry, <sup>2</sup> Department of Internal Medicine II, Klinikum Grosshadern, Ludwig-Maximilians-Universität Munich, D-81377 Munich, Germany; \* author for correspondence: fax 49-7095-3240, e-mail Michael.Vogeser@klch.med.uni-muenchen.de)

The measurement of adrenocorticotrophic hormone (ACTH 1-39;  $M_r$  4500) plays a key role in the evaluation of hyper- and hypocortisolism (1–3), but low ACTH concentrations make accurate measurement challenging. Two automated, nonisotopic ACTH assays have become available in recent years. Because it is usual for rather small series to be assayed for ACTH in a clinical setting and because nonisotopic methods with stable calibration are of great practical interest, we compared these assays.

Both assays investigated were solid-phase, sandwich immunoassays that use chemiluminescence for signal generation and are implemented on benchtop, multichannel, random-access analyzers with ready-to-use reagents.

The Nichols Advantage ACTH assay (Nichols Institute Diagnostics) uses one acridinium ester-labeled mouse monoclonal antibody that specifically binds to the C-terminal region of ACTH and one biotin-labeled goat polyclonal antibody that binds to the N-terminal region. The sample is incubated for 21 min at 37 °C with both antibodies simultaneously, leading to the formation of a soluble sandwich complex in the presence of ACTH molecules. Streptavidin-coated magnetic particles are then added, and the reaction mixture is incubated for 10 min, during which the sandwich complex binds to the particles by biotin-avidin interaction. The particles are fixed to the wall of the single-use reaction cell magnetically, and unbound, labeled antibody is separated by aspiration and subsequent washing. Finally, two trigger solutions are injected into the reaction cells to initiate the chemiluminescent reaction, and the light emission is quantified over 2 s by a luminometer. A lot-specific master calibration curve is loaded by barcode and adjusted weekly with two calibrators by the user. The reported measuring range extends to 1500 ng/L. Barcode-labeled sample tubes are processed directly. The sample throughput is ~90/h, and the time to first result is 37 min with a start-up time of ~15 min.

In the DPC IMMULITE ACTH assay (Diagnostic Products Corporation), a polystyrene bead within a test unit serves as the solid phase; the bead is coated with a monoclonal murine anti-ACTH antibody. The patient sample and a buffer are introduced and incubated for 30 min at 37 °C with intermittent agitation of the test unit. Unbound sample is then removed by a centrifugal wash procedure. Subsequently, an alkaline phosphatase-labeled polyclonal rabbit anti-ACTH antibody is introduced for a second 30-min cycle, after which unbound enzyme conjugate is removed by another centrifugal wash. Finally, the chemiluminescent substrate, a phosphate ester of adamantyl dioxetane, is added. This compound under-

goes hydrolysis in the presence of alkaline phosphatase to yield an unstable intermediate, producing a sustained emission of light that is photomultiplied and quantified for 12 s. Adjustment of a predefined, lot-specific master calibration curve is performed with two calibrators at recommended intervals of 2 weeks. The reported measuring range is 10–1250 ng/L. The system reads sample barcodes after an aliquot of the sample has been pipetted into an assay cup and the test units have been placed with the cups on a load chain. The sample throughput is ~90 determinations per hour; the time to first result is 70 min with a start-up time of 10 min.

Both assays were handled according to the manufacturers' instructions in all respects by experienced technicians in a routine laboratory setting. Quality control was performed for all analytical runs using lyophilized control materials provided by the respective manufacturers (two concentrations for the DPC assay and three for the Nichols assay) according to the German Medical Association guidelines. The study protocol was approved by the institutional review board.

Linearity of the assays was investigated by serial dilution of a high-ACTH patient sample (>1250 ng/L in the DPC assay and 1110 ng/L in the Nichols assay) with a low-ACTH pool in nine steps up to a dilution of 1:512. In both assays, results were linear ( $r^2 > 0.99$ ).

To study the interassay imprecision of the assays, four plasma pools were prepared from residual patient samples, aliquoted after equilibration, and then stored at –70 °C. ACTH was measured in 12 analytical runs using two different lots of reagents and five calibration cycles in both assays over a 3-month period. After thawing at 4 °C, the samples were centrifuged and split for analysis by both assays simultaneously; each sample was analyzed once in each assay. The results of the imprecision study are given in Table 1.

For the method comparison study, 444 clinical EDTA-plasma samples were used. In 42 of the samples, ACTH values determined with the DPC and Nichols assays were <10 ng/L; in 17 samples, ACTH was >10 ng/L with the DPC assay and <10 ng/L with the Nichols assay; in 37 samples, ACTH was <10 ng/L with the DPC assay and >10 ng/L with the Nichols assay. Results for the remaining 348 samples were compared by unbiased regression analysis according to Deming: Nichols = 0.81(DPC) + 13.3 ng/L; 95% confidence interval for slope, 0.79–0.82; 95% confidence interval for intercept, 11.9–14.6 ng/L (Pearson  $r = 0.982$ ).

**Table 1. Interassay imprecision of the DPC IMMULITE and the Nichols Advantage ACTH assays (n = 12 days over 3 months).**

Pool	DPC IMMULITE		Nichols Advantage	
	Mean ACTH, ng/L	CV, %	Mean ACTH, ng/L	CV, %
1	<10		4.7	37
2	<10		9.9	15
3	15.7	11	17.7	12
4	811	2.8	731	6.1

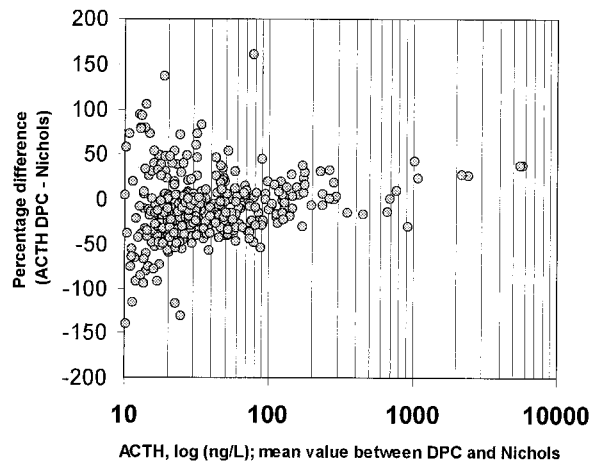


Fig. 1. Comparison of the DPC Immulite and the Nichols Advantage ACTH assays: difference plots with percentage of difference vs mean concentration ( $n = 348$ ).

Mean concentrations of all analyzed samples were 80.3 and 83.1 ng/L with the Nichols and DPC assays, respectively (medians, 33.9 and 30.4 ng/L, respectively). For all paired results with a mean concentration between 10 and 50 ng/L by both assays ( $n = 223$ ), regression analysis yielded: Nichols =  $1.10(\text{DPC}) + 0.1 \text{ ng/L}$ ; 95% confidence interval for slope, 1.00–1.20; 95% confidence interval for intercept,  $-2.3$  to  $2.5 \text{ ng/L}$  ( $r = 0.71$ ).

Further comparisons of all results  $>10 \text{ ng/L}$  using the method described by Bland and Altman (4) demonstrated a mean difference of  $-7\%$  (DPC minus Nichols) with a SD of  $29\%$  (Fig. 1).

Of the samples used for the method comparison, 356 were obtained from 89 patients during corticotropin-releasing hormone (CRH) stimulation testing. For this test,  $100 \mu\text{g}$  of recombinant CRH (CRF Ferring<sup>®</sup>; Ferring) was given intravenously, and samples were drawn at  $-15$ ,  $0$ ,  $30$ , and  $60 \text{ min}$ . Individual absolute increments of ACTH concentrations during CRH stimulation tests were compared between the two assays by Deming regression analysis [baseline concentration subtracted from stimulated concentration at  $30$  and  $60 \text{ min}$ , respectively ( $\ln 30$  and  $\ln 60$ )]:  $\ln 30 \text{ Nichols} = 0.99 \times \ln 30 \text{ DPC} + 1.5 \text{ ng/L}$ ; 95% confidence interval for slope,  $0.94$ – $1.04$ ; 95% confidence interval for intercept,  $-0.8$  to  $3.7 \text{ ng/L}$ ;  $r = 0.970$ ; and  $\ln 60 \text{ Nichols} = 1.04 \times \ln 60 \text{ DPC} - 0.5 \text{ ng/L}$ ; 95% confidence interval for slope,  $0.97$ – $1.10$ ; 95% confidence interval for intercept,  $-2.3$  to  $1.3 \text{ ng/L}$ ;  $r = 0.954$ .

Apparently discordant results between the two assays were found in two patients.

Patient 1 was a 19-year-old woman with septo-optical dysplasia, diabetes insipidus, and amaurosis. Her admission was for clinical signs of hypocortisolism. In the CRH stimulation test, her ACTH results at  $-15$ ,  $0$ ,  $30$ , and  $60 \text{ min}$  were  $40.3$ ,  $35.6$ ,  $96.8$ , and  $111 \text{ ng/L}$ , respectively, for the Nichols assay, and  $<10$ ,  $<10$ ,  $59.5$ , and  $64.0 \text{ ng/L}$  for the DPC assay. Serum cortisol concentrations (DPC IMMULITE Cortisol assay) at the same times were  $52$ ,  $44$ ,  $63$ , and  $72 \text{ nmol/L}$ .

Patient 2 was a 51-year-old woman with adrenomyeloneuropathy. Her admission was for exclusion of adrenocortical insufficiency, which may occur in adrenomyeloneuropathy. Her unstimulated basal ACTH was  $15.0 \text{ ng/L}$  for the Nichols assay and  $140 \text{ ng/L}$  for the DPC assay; her serum cortisol concentrations were  $522$  and  $834 \text{ nmol/L}$  before and after stimulation with exogenous ACTH.

The explanation for these discrepant results is not known. All determinations were repeated from the original tubes to exclude benchwork and clerical errors. Heterophilic antibodies may cause such discrepancies.

To assess the manufacturers' stated reference intervals, basal morning ACTH was determined in 20 healthy volunteers. The central 95% interval was  $13$ – $57 \text{ ng/L}$  (mean,  $27 \text{ ng/L}$ ; median,  $21 \text{ ng/L}$ ) for the DPC assay (95% reference interval given by DPC  $<10$ – $46 \text{ ng/L}$ ; median,  $24 \text{ ng/L}$ ;  $n = 59$ ) and  $15$ – $46 \text{ ng/L}$  (mean,  $29 \text{ ng/L}$ ; median,  $28 \text{ ng/L}$ ) for the Nichols assay (95% reference interval given by Nichols,  $9$ – $52 \text{ ng/L}$ ; mean,  $22 \text{ ng/L}$ ;  $n = 99$ ).

The possible influence of typical interferences on ACTH determination was studied by dilution and recovery experiments. Pooled plasma from uremic patients (creatinine,  $681 \mu\text{mol/L}$ ; urea,  $36.7 \text{ mmol/L}$ ) was diluted (1 volume + 1 volume and 1 volume + 3 volumes) with a low-ACTH pool (creatinine,  $62 \mu\text{mol/L}$ ; urea,  $5.3 \text{ mmol/L}$ ). ACTH as measured in the two pools and in the diluted samples in duplicate was subjected to linear regression analysis. In the same way, the possible influence of hyperbilirubinemia was studied (bilirubin,  $457$  and  $6.8 \mu\text{mol/L}$  in the high and low pools, respectively). ACTH results exhibited linearity in both experiments ( $r > 0.95$ ), making relevant interference unlikely. To study the influence of lipemia, we added a lipid-rich solution for parenteral nutrition (Lipofundin N 20%; B. Braun) to a plasma pool with a triglyceride concentration of  $20 \text{ g/L}$ . ACTH concentrations were determined in duplicate in the native and treated samples; after correction for volume dilution ( $70 \mu\text{L}$  of solution plus  $1000 \mu\text{L}$  of sample),  $94\%$  of the original concentration was found by the DPC assay and  $82\%$  by the Nichols assay. Addition of hemolyzed erythrocyte suspension ( $10 \mu\text{L}$  plus  $1000 \mu\text{L}$  of sample; final hemoglobin concentration,  $1.88 \text{ g/L}$ ) led to a lower mean ACTH concentration with the DPC assay ( $83\%$ ) and a higher concentration with the Nichols assay ( $143\%$ ) compared with the native sample.

No carryover effect could be demonstrated in either assay using a sample with a low ACTH concentration ( $<10 \text{ ng/L}$  by both assays) analyzed in quadruplicate, in each case pipetted immediately after a sample with a mean ACTH of  $1326 \text{ ng/L}$ .

In routine use, the mechanical components of the IMMULITE analyzer (with only one rotating pipetting arm) were rugged. The Nichols Advantage analyzer allowed convenient workflow, with host-data download and no manual pipetting, but several mechanical malfunctions occurred during the study.

Reproducibility of these automated, short-incubation

tests was slightly inferior to that reported for an IRMA with a CV of 9.3% at a concentration of 8.7 ng/L ( $n = 20$ ) (5). This probably reflects the distinctly shorter incubation times of the automated assays (at a temperature of 37 °C vs ambient temperatures in manual assays, which may allow analyte degradation). Also using the Nichols assay reporting results <10 ng/L precise determination of sub-normal ACTH concentrations is possible only in a narrow range below the lower limit of the reference range for unstimulated morning ACTH.

Comparability of the ACTH results obtained by the two automated assays under investigation was found to be limited for individual samples within the reference range for basal ACTH. ACTH increments during CRH simulation testing, however, showed close correlation, yielding identical diagnostic information in most cases and similar reference ranges. Nevertheless, discrepancies of results must be expected in a relevant number of tests when these different ACTH assays are used, possibly because of heterophilic antibodies. We speculate that the limited overall comparability may be attributable to different cross-reactivities of the assays with related molecules with longer half-lives, e.g., proopiomelanocortin. In the DPC ACTH assay, the analyte-antibody sandwich is formed in two separate incubations with a wash step, compared with a one-step incubation with both antibodies in the Nichols assay. The latter approach may produce less specific binding and might explain the constant and proportional bias between the two assays, with lower concentrations found with the DPC assay.

We conclude that the use of automated ACTH assays represents progress in the clinical laboratory setting. Either of the assays investigated here can allow cost-efficient analysis near the site of patient care without the need for sample shipment on dry ice to larger laboratories. This will allow straightforward evaluation without the delays in obtaining laboratory reports that might increase the overall cost of treatment.

## References

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6. **Rapid Homogeneous Immunoassay for Human Ferritin in the Cobas Mira Using Colloidal Gold as the Reporter Reagent**, Patrick Englebienne,<sup>1\*</sup> Anne Van Hoonacker,<sup>2</sup> and Joseph Valsamis<sup>3</sup> (Departments of <sup>1</sup> Nuclear and <sup>3</sup> Laboratory Medicine, Free University of Brussels, Brugmann University Hospital, Place van Gehuchten 4, B-1020 Brussels, Belgium; <sup>2</sup> Englebienne & Associates, Strijpstraat 21, B-9750 Zingem, Belgium; \* author for correspondence: fax 32-9-384-7250, e-mail patrick.englebienne@skynet.be)

Serum ferritin concentrations, with some exceptions (1–3), reflect iron stores (4, 5). Ferritin assays must have a broad dynamic range because the serum concentrations can be <10 µg/L (6) or >1 mg/L in some types of malignancies (7). Radio- and enzyme immunoassays have been used routinely (8), but rapid, automated latex agglutination immunoassays have been developed and validated (9–11). These methods have drawbacks (12), of which the disturbance of colloidal stability by nonspecific bridging processes particularly should be avoided (13).

In our quest for new reporter reagents (14, 15), our attention was drawn to colloidal gold as a potential substitute for latex in particle-enhanced agglutination immunoassays. Colloidal gold was used by Leuvering et al. (16) in sol particle immunoassays (SPIAs) for several serum or urine analytes. Unfortunately, the technique was prone to interference when undiluted serum samples were used (17). Recently, we showed (18, 19) that the change in visible absorbance at 600 nm ( $A_{600}$ ) observed when colloidal gold particles coated with an antibody interact with the antigen results not only from agglutination but also from subtle changes in the refractive index at the particle surface [surface plasmon resonance effect (SPR)]. Thus, the unidentified random interferences noted by Leuvering et al. (16) could result from interactions between nonspecific reacting sites on the antibody molecule (distinct from the binding site) and several serum components (distinct from the analyte). According to this model, these interactions were transduced as a photometric signal by the SPR effect of gold (19, 20), which adds to the signal produced by the agglutination. With this in mind, we optimized the buffer to be used in the SPIA with colloidal gold and carefully selected the antibodies to be used for their lack of SPR effects with human serum components other than the analyte. We present here the results obtained with an assay for human serum ferritin.

We synthesized colloidal gold particles with a mean diameter of 50 nm by reducing a boiling aqueous hydrogen tetrachloroaurate solution (500 mL, containing 0.4 g/L hydrogen tetrachloroaurate) with 20 mL of a 10 g/L solution of sodium citrate. The gold sol was then adjusted to pH 9 and coated separately during mixing for 10 min under magnetic stirring with two murine monoclonal antibodies (057-10030 and 090-12710; OEM Concepts) against ferritin. The antibodies, previously diluted in water (50 mg/L), were added rapidly to the colloidal gold sol to a final antibody concentration of 15 mg/L. These antibodies were selected because of their lack of reactivity with nonspecific human serum components by SPR (19).