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 subnormal 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

Rapid Homogeneous Immunoassay for Human Ferritin in the Cobas Mira Using Colloidal Gold as the Reporter Reagent, Patrick Englebienne,1* Anne Van Hoonacker,2 and Joseph Valsamis3 (Departments of 1 Nuclear and 3 Laboratory Medicine, Free University of Brussels, Brugmann University Hospital, Place van Gehuchten 4, B-1020 Brussels, Belgium; 2 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 (ΔA600) 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).
The minimum amount of IgG and the optimal pH for coating the particles by charge adsorption were determined by isothermal titration \((19, 20)\). Such preliminary optimization allowed us to use an IgG concentration sufficient to saturate the particle surface; consequently, neither subsequent centrifugations nor washings were required. The coated particles were then buffered at pH 9.0 and stabilized by the sequential addition of sodium tetraborate \((50 \text{ mmol/L})\), sodium chloride \((150 \text{ mmol/L})\), bovine serum albumin \((5 \text{ g/L})\), sodium azide \((0.9 \text{ g/L})\), and Tween 20 \((2 \text{ mL/L})\). The two gold-antibody conjugates were then mixed at a ratio of three parts of 090-12710 conjugate per one part of 057-10030 conjugate. Once buffered, the reagent could be used immediately. By contrast, the preparation of latex reagents would require extensive centrifugation and washing steps to eliminate unbound or loosely bound IgG \((21, 22)\). The further addition of bovine serum albumin and Tween after buffering at high pH prevented nonspecific bridging between particles.

On the Cobas Mira, we used a reaction mode with sequential additions of sample \((30 \mu\text{L})\) and reagent \([120 \mu\text{L} \text{ of accelerating buffer containing } 200 \text{ mmol/L Tris-HCl (pH 8.0), } 70 \text{ g/L poly(ethylene glycol) 6000, } 15 \text{ g/L poly-}
(\text{ethylene glycol) 35 000, } 0.9 \text{ g/L sodium azide, and } 10 \text{ mL/L Triton X-100]}\), followed by mixing and the addition of 50 \(\mu\text{L} \text{ of gold-antibody conjugate. Because the viscos-
ities of the sample and reagents were substantially differ-

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**Fig. 1.** Changes in \(A_{600}\) during incubation time \((A)\) and typical dose–response relationship obtained in optimized assay conditions \((B)\).

\((A)\), normalized changes (blank subtracted) in \(A_{600}\) during incubation time were observed after addition (arrow) of the gold-antibody conjugate to mixtures of buffer and calibrators containing 0 \((\Delta)\), 10 \((\odot)\), 50 \((\bigcirc)\), 150 \((\diamondsuit)\), 300 \((\blacksquare)\), and 600 \((\lozenge)\) \(\mu\text{g} \text{ of human ferritin/L. For comparison, the kinetics of a sample containing a ferritin concentration above the equivalence point (2000 \(\mu\text{g/L})\) is also displayed \((\star)\). \((B)\), the signal data are the means ± SD \((\text{bars})\) from three independent curves in duplicate.

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**Table 1.** Correlations between the colloidal gold assay \((y)\) and the Chiron Diagnostics ACS:180 or the Roche Unimate latex assay \((x)\) for human ferritin.

<table>
<thead>
<tr>
<th>Comparative technique ((x))</th>
<th>(n)</th>
<th>Correlation equation</th>
<th>(r)</th>
<th>Correlation coefficient</th>
<th>Average bias, (\mu\text{g/L})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chiron Diagnostics ACS:180</td>
<td>112</td>
<td>(y = 1.02x + 0.5)</td>
<td>0.016</td>
<td>0.99</td>
<td>2.65</td>
</tr>
<tr>
<td>Roche Diagnostics Unimate Latex</td>
<td>103</td>
<td>(y = 1.18x - 14.9)</td>
<td>0.035</td>
<td>0.96</td>
<td>4.09</td>
</tr>
</tbody>
</table>
ent and to improve repeatability, we added a small volume of water (2 μL by pipetting) immediately after the addition of the gold-antibody conjugate, so that the instrument mixed the cuvette content a second time. Using the optimal sample:reagent ratio, we tested the addition of several components to the buffer to improve the signal specificity (18, 19). Magnesium chloride, mannitol, and EDTA were each found to substantially improve the signal specificity at concentrations up to 0.25, 0.11, and 0.01 mol/L, respectively. During the kinetics of interaction, which occurred after addition of the gold-antibody conjugate (Fig. 1A), the A₆₀₀ changes were quick and linear during the first 2 min; consequently, we limited the specific reaction (sample-reagent-gold conjugate) incubation time to 2.08 min (5 cycles of 25 s). Because of the good linearity of the kinetics, we programmed the instrument in kinetic mode, which allowed us to report the response as change in A₆₀₀ per min. Fig. 1B displays the mean (± SD) of three calibration curves performed in duplicate in the optimized conditions.

With this optimized assay, the least detectable concentration calculated at 3 SD above the zero calibrator was 4.5 μg/L. A Heidelberger-Kendall curve performed up to 6000 μg/L indicated that the equivalence point (hook effect) was >1200 μg/L. The kinetic reading mode of the assay allowed the identification of samples with very high ferritin contents. Such samples showed an earlier plateau and a marked increase in A₆₀₀ during the first 25 s of incubation (Fig. 1A), which was well above that of the highest calibrator. Thus, the program included a ΔA₆₀₀ threshold for the first 25 s above which the instrument reassayed samples automatically after proper dilution to avoid hook effect errors encountered with similar sandwich immunoassays for ferritin (23).

Assay precision was evaluated according to the NCCLS protocol (24) using three control sera (52, 123, and 334 μg/L ferritin, respectively). Within-run, between-run, between-day, and total imprecisions (CVs) were 1.5–4.3%, 2.3–3.8%, 3.5–5.3%, and 4.8–7.9%, respectively. These results were similar to those observed with latex immunoassays for ferritin (9, 10).

Recovery with liver ferritin added to serum (62–3239 μg/L; n = 8) was 90.7–109.5% (mean ± SD, 100.1 ± 6.3%), which compared favorably with latex (10) and ELISA (25) assays.

We evaluated interferences according to NCCLS and Société Française de Biologie Clinique (20) protocols at three ferritin concentrations. Bilirubin did not interfere up to the highest concentration tested (300 mg/L). Similarly, no interferences were observed from the common anticoagulants heparin (up to 0.5 g/L), EDTA, sodium citrate (10 g/L), and sodium fluoride (up to 5 g/L). Hemoglobin concentrations >2 g/L produced a positive linear interference. The major interference found with this assay was exerted by lipids. The interference was particularly marked for low-concentration samples. A similarly substantial lipid interference requiring the pretreatment of turbid samples by centrifugation or with a lipid-clearing agent has been reported for a commercially available latex-based reagent (10). IgM rheumatoid factor (RF) also exerted a substantial positive interference in the assay. This could be expected because the gold particles were coated with complete murine antibody IgG molecules. This latter type of interference reportedly could be avoided by either coating the gold particles with the F(ab')₂ fragments instead of the complete IgG (26) or by use of a polyclonal antiserum against RF (27). The RF interference was greatest at low ferritin concentrations. This may indicate that, at higher analyte concentrations, the ferritin-antiferritin agglutination process impairs RF access to the Fc fragment of the antibodies.

We compared the results of unknown samples observed with this new technique to those obtained with the ACS:180 technique from Chiron Diagnostics (chemiluminescence) and the Unimate latex reagent from Roche Diagnostics (Table 1). The residual plot did not indicate a substantial difference for the ACS. Results were higher with the colloidal gold than with the Unimate (slope, 1.18; 5₀₅₀ = 0.035), mainly because of a single sample that for which the measured concentration was more than twice that observed with latex. This discrepancy was not observed in the ACS:180 correlation, and both results for the same sample were in agreement. Because the results obtained with the ACS:180 method, which was not based on particle agglutination, fully agreed with the colloidal gold results, we suggest that the discrepancy resulted primarily from differences in epitope recognition by the antibodies used in the agglutination techniques or in the tissue ferritin used in the different calibrators, as noted recently (28), rather than from a nonspecific interaction with the gold probes. This is further supported by the fact that identical discrepancies were observed during an evaluation of this gold reagent in an independent laboratory, involving comparative studies with the Unimate latex and another latex reagent of commercial origin. With the outlier excluded, the regression slope was 1.10 (5₀₅₀ = 0.028) and the mean bias was 0.95 μg/L.

Despite the random and unidentified interference problems identified in the early days of its use as the reporter reagent in SPIA (17), colloidal gold is likely to constitute an interesting alternative to latex in homogeneous immunoassays. We have demonstrated that the reagent is applicable to a ferritin homogeneous immunoassay. This was made possible by reagent optimization, which eliminated the nonspecific interactions that could lead to a plasmon-resonance effect (18, 19) and interfere with the signal resulting from the specific agglutination of the antibody-sensitized particles. However, for the interference with RF to be circumvented (26, 27), further investigation and assay validation with samples containing other autoantibodies or myeloma paraproteins will be required. The colloidal particles are much easier to synthesize and to coat by charge adsorption with the suitable antibodies than latex (20). A recent report (21), which compared two covalent-coating procedures with one passive adsorption procedure for antibody immobilization on latex particles, concluded that, despite the centrifugation and washing steps involved, substantial aggregation...
occurred. Even when latex particles with sizes similar to colloidal gold were used for reagent preparation to decrease the sedimentation rate during storage, expensive synthetic materials and tedious coating procedures were involved, including several ultracentrifugation and sonication steps (22). Once coated and properly stabilized, the colloidal gold particles did not show any sign of nonspecific agglutination. To date, we have used reagent preparations stored at 4°C for >20 months without any substantial loss in reactivity. The performance of the ferritin assay reported here compares at least equally with other immunoassay technologies, including latex, ELISA, and chemiluminescence.

Our experience with the colloidal gold reagent in immunoassay points to other important advantages that further ease preparation compared with latex. In particular, application of the technology to other analytes, such as the human cardiac fatty acid-binding protein (18, 19), has shown that the detection limit that could be reached was ~1 log-unit lower than the detection limit achievable with a matching latex reagent.

We acknowledge the support of Roche Diagnostic, which provided the latex reagent and a Cobas Mira instrument for the performance of the correlation studies. We are also grateful to Drs. Eisenwiener, Dessauer, Karl, and Kürtzinger of Roche Diagnostics Research and Development for the fruitful discussions and their permission to publish the results of the correlation studies.

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Quantification of hTERT mRNA and Telomerase Activity in Bladder Washings of Patients with Recurrent Urothelial Cell Carcinomas, Jacques B. de Kok,1 Michael R. van Balken,2 Rian W.H.M. Roelofs,1 Yvonne A.W.G. van Aarssen,3 Dorine W. Swinkels,1 and Jacqueline M.T. Klein Gunnewiek1

In patients with superficial urothelial cell carcinomas (UCCs), 80% of tumors recur after transurethral resection. A minority (10–20%) of these recurrent tumors will progress to high-grade and muscle-invasive disease (1). Prediction of progression would enable the urologist to determine individual therapy to reduce mortality.

For the detection of recurrent disease, endoscopic evaluation (cystoscopy) is considered the gold standard (1). Cystoscopy is supplemented with urine or bladder wash