served not only decreases in FA concentrations (assumingly attributable to FA peroxidation), but also increases. A second process may be ongoing during storage: the formation of erythrocyte microparticles. In vitro, glycolysis will lower the glucose concentration in erythrocytes, causing depletion of ATP. Consequently, the erythrocytes cannot maintain membrane integrity, and microparticles will be released (15). Over time, the concentration of plasma phospholipid-associated FAs may thus become enriched with erythrocyte membrane phospholipids containing relatively high amounts of polyunsaturated FAs.

Our study has some limitations. One limitation is that we did not measure in duplicate to adjust for intraassay variation. In addition, serum samples were not analyzed within one run to minimize interassay variability. However, standardized procedures were used for analysis, with CVs ≤7.9% for serum analytes and ≤5.6% for plasma phospholipid-associated FAs. In addition, the reliability analyses showed high agreement between follow-up and baseline values. Another limitation is that samples were not randomized before analysis. Possible bias from order of draw, although unlikely, can therefore not be ruled out. A third limitation is that we were not able to test the reliability and validity of CRP over the 28-h storage period. However, changes in concentration were small (<5.5%), which, as mentioned before, is in line with results from other studies (3, 5).

In conclusion, this study shows that in the context of epidemiologic studies investigating (nutritional) status during routine care, a pragmatic approach to blood collection may validly be applied to determine CRP, retinol, ferritin, folic acid, or FA status. Although storage will diminish the precision of estimates, standard (correlational) epidemiologic analyses will not be compromised in samples stored for a maximum of 28 h.

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References


Development and Validation of an Automated Chemiluminesmetric Immunoassay for Human Intrinsic Factor Antibodies in Serum, Elizabeth A. Gomez,1 Daniel D. Ehresmann,1 Lisa K. Ledebuhr,2 Mary L. Eastvold,2 Ravinder J. Singh,2 George G. Klee,2 and Stefan K.G. Grebe2,3 (1 Beckman Coulter Inc., Chaska, MN; Departments of 2 Laboratory Medicine and Pathology and 3 Medicine, Mayo Clinic, Rochester, MN; * address correspondence to this author at: Endocrine Laboratory, Hilton 730C, Mayo Clinic, 200 1st Street SW, Rochester, MN 55905; fax 507-284-9758, e-mail grebs@mayo.edu)

The cobalamin, also referred to as vitamin B12, are a group of closely related enzymatic cofactors involved in the conversion of methylmalonyl-coenzyme A to succinyl-coenzyme A and in the synthesis of methionine from homocysteine (1–3). Vitamin B12 deficiency can lead to megaloblastic anemia and neurologic deficits (4). The latter may exist without anemia or precede it. Adequate replacement therapy will generally improve or cure cobalamin deficiency. Unfortunately, many other conditions, which require different interventions, can mimic the symptoms and signs of vitamin B12 deficiency (4). Moreover, even when cobalamin deficiency has been established, clinical improvement may require different dosages or routes of vitamin B12 replacement, depending on the underlying cause (3, 4). In particular, patients with pernicious anemia, possibly the commonest type of cobalamin deficiency in developed countries, require either massive doses of oral vitamin B12 or parenteral replacement therapy (3–5). The reason is that in pernicious anemia, patients suffer from gastric mucosal atrophy, which leads to diminished or absent gastric acid, pepsin, and intrinsic factor (IF) production. Because gastric acid,
pepsin, and IF are required for liberation of cobalamin from binding proteins and efficient cobalamin absorption in the gut, respectively, vitamin B$_{12}$ deficiency ensues \((3, 4)\). Most of these patients have autoantibodies against gastric parietal cells or IF, with the latter being very specific but present in only \(\sim 50\%\) of cases \((1)\).

The aim of the work-up of patients with suspected vitamin B$_{12}$ deficiency is therefore to confirm the presence of deficiency and to establish its most likely etiology. Many different testing algorithms have been proposed to achieve maximum sensitivity and specificity for both tasks, but almost all involve measurement of serum vitamin B$_{12}$ and IF antibodies (IFABs) at some step \((1, 2, 4, 6)\). Given that the prevalence of undiagnosed cobalamin deficiency approaches \(2\%\) in the elderly, both assays are high-volume tests \((7)\). This represents no problem with regard to vitamin B$_{12}$ measurements because there are many different automated assays available. To date, however, all IFAB tests have been manual immunoassays. In the following, we describe the development and validation of an automated IFAB assay.

The Beckman Access® vitamin B$_{12}$ immunoassay first liberates protein-bound B$_{12}$ in patient samples, followed by competition of vitamin B$_{12}$ and a solid-phase monoclonal antibody against the IF-binding site for labeled IF. We realized that this assay format could be modified to allow detection of IFABs instead of vitamin B$_{12}$. In the modified assay, IFABs in patient samples compete with monoclonal B$_{12}$-binding site IFABs for enzyme-labeled IF, as illustrated in Fig 1. We prevent interference from vitamin B$_{12}$ by omitting the initial step of liberating protein-bound cobalamin and by adding a monoclonal mouse antivitamin B$_{12}$ antibody to neutralize free cobalamin (McxB12; Sigma-Aldrich). This is important because patients are often given vitamin B$_{12}$ before being tested for IFABs. Vitamin B$_{12}$ concentrations \(\geq 750\) pmol/L \((\sim 1000\) ng/L) can saturate serum B$_{12}$-binding proteins \((8)\), leading to increased concentrations of free cobalamin and, potentially, false positive IFAB tests \((9)\). The assay signal generated is inversely proportional to the concentration of IFABs in a patient sample and is expressed in antibody units (AU)/mL. One AU is defined as the maximum possible bound signal and should correspond to the absence of any IFABs in a patient’s serum. Results are reported in AU/mL and as negative, equivocal, or positive, based on cutoffs established during clinical validation (see below).

Assay validation included \((a)\) intra- and interassay imprecision; \((b)\) dilution linearity; \((c)\) assay interference by hemoglobin, bilirubin, triglycerides, serum albumin, and vitamin B$_{12}$; \((d)\) establishment of healthy-population ref-

![Fig. 1. Schematic depiction of the Access IFAB assay.](image-url)

Solid-phase anti-IFAB complexes compete with IFABs in patient samples for alkaline-phosphatase-labeled intrinsic factor (ALP-IF) contained in the assay reagents. Although transcobalamin-bound vitamin B$_{12}$ (TC-bound B$_{12}$) in patient samples does not interfere, free B$_{12}$ could bind to ALP-IF. This is prevented by the addition of anti-B$_{12}$ antibodies (Anti-B$_{12}$ AB) to the reagent mixture. After separation of unbound ALP-IF from the solid-phase anti-IFAB complexes, chemiluminescent alkaline phosphatase substrate is added. The IFAB concentration contained in the patient sample is inversely proportional to the generated light intensity.
The observed IFAB values ranged from 0.93 to 50.82 AU/mL. In the Access IFAB assay, the minimum free vitamin B12 concentrations were between 1.1–392.6 pmol/L (1.5–530 ng/L), demonstrating that in the Access IFAB assay the minimum free vitamin B12 concentration range of 0.93–12.25 AU/mL, with the respective diagnostic cutoff of 172.6 pmol/L (233 ng/L).

The reference interval for a healthy population (n = 200; 50% male and 50% female) was 0.93–1.20 AU/mL. The positive cutoff value for the Access IFAB Assay was determined by ROC analysis of 499 samples across a IFAB concentration range of 0.93–12.25 AU/mL, with the results of the DPC RIA used as the comparison method. The raw AU data showed a log (Access) to linear (DPC) relationship as depicted in Fig. 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue1/. The area under the ROC curve for the Access IFAB assay was 0.947. Values within the reference interval were categorized as negative. We selected 1.53 AU/mL as our positive cutoff, the point where the maximum sensitivity and specificity (~87% for each) were obtained. Values between the upper limit of the reference interval (1.20 AU/mL) and 1.53 AU/mL were designated as equivocal. This classification mirrors that used in the DPC RIA, which also has an equivocal range between its upper reference interval limit and its positive diagnostic cutoff.

To validate the two diagnostic cutoffs for abnormal results, we re-assayed, in a blinded fashion, 127 consecutive patient samples sent for diagnostic vitamin B12 testing with both the DPC RIA and the Access IFAB assay. The observed IFAB values ranged from 0.93 to 50.82 AU/mL in the Access IFAB assay. Results for each assay were classified by their respective cutoffs as negative, equivocal, or positive. The two diagnostic categorizations by the Access IFAB assay and the DPC RIA were compared by weighted κ testing (Table 1). Diagnostic agreement between the assays was excellent. The overall weighted κ was 0.90 (95% confidence interval, 0.84–0.96), i.e., 90% agreement in classification of samples. The κ values for the individual categories were 0.87 (0.79–0.96) for negative IFAB results, 0.41 (0.09–0.73) for equivocal results, and 0.94 (0.87–1.00) for positive results. There was insufficient serum for further characterization of the two samples that were classified as negative by the Access IFAB assays but positive by the DPC RIA.

In the separate group of patients with autoimmune diseases or known presence of heterophile antibodies/HAMAs, the two assays showed 96.6% diagnostic agreement. All samples [24 with HAMAs, 25 with heterophilic antibodies, 5 from patients with diabetes, 5 from patients with Graves disease, and 14 from patients with other thyroid disorders (5 from patients with Hashimoto disease, 5 containing thyroid autoantibodies, and 4 from patients with hyperthyroidism of unspecified cause)] were negative in both methods tested. Of 14 rheumatoid arthritis samples, 5 were positive in the Access IFAB assay. Of these, two were also positive in the DPC RIA. The three discrepant samples were further characterized as IFAB positive by testing with the Genesis Diagnostics IFAB ELISA or by demonstrating inhibition by added IF in the Access IFAB assay.

We were able to develop an automated IFAB assay with relative ease by modifying an existing automated vitamin B12 assay. It appears that the new assay has diagnostic performance similar to that for the DPC RIA, which has performed well with regard to sensitivity and specificity in previous studies comparing different commercial and noncommercial IFAB assays (10). However, an optimized IFAB assay has been described (11), which may offer higher detection sensitivity than the DPC RIA and, by implication, possibly the Access IFAB assay. Similarly, detection of type II IFABs, which are not directed against the B12-binding pocket of IF, may improve diagnostic sensitivity (12). Detection of type II antibodies has not been characterized for the Access IFAB assay but may be possible because they could prevent the labeled IF from binding to the solid phase through steric hindrance.

Although there is ongoing debate about the diagnostic sensitivity of particular IFAB assays, they are all generally

<table>
<thead>
<tr>
<th>Diagnostic category by Access IFAB</th>
<th>Negative</th>
<th>Equivocal</th>
<th>Positive</th>
<th>Total</th>
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<tr>
<td>Negative</td>
<td>61</td>
<td>6</td>
<td>2</td>
<td>69</td>
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<tr>
<td>Equivocal</td>
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<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
<td>2</td>
<td>53</td>
<td>55</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>11</td>
<td>55</td>
<td>127</td>
</tr>
</tbody>
</table>

Table 1. Diagnostic agreement between the Access IFAB assay and the DPC IFAB RIA.
regarded as very specific (1, 6). Because of this specificity, a proportion of patients who are investigated for vitamin B$_{12}$ deficiency will end up being tested for the presence of IFABs, regardless of the diagnostic cascade favored by the investigating physician(s) (1, 2). In referral laboratories, this may lead to substantial test volumes of labor-intensive manual RIAs, with all the associated problems. These range from an increased risk of sample mix-ups or analytical mishaps attributable to multiple manual sample-handling steps, to difficulties in maintaining consistent performance, to the need to handle and dispose of radioactivity, and to lower analytical precision and increased turnaround time compared with automated assays. The new automated IFAB assay addresses all of these problems while maintaining comparable diagnostic accuracy. Moreover, both vitamin B$_{12}$ measurements and IFAB measurements can be performed on the same instrument, with the possibility to tag IFAB testing to individual measurements can be performed on the same instrument, with the possibility to tag IFAB testing to individual vitamin B$_{12}$ samples, conditional on the measured cobalamin concentration, a further improvement in workflow. We therefore believe that the availability of this new IFAB assay provides an opportunity for increased and more expeditious testing and diagnosis of pernicious anemia.

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References


Diagnosis of Methylmalonic Acidemia from Dried Blood Spots by HPLC and Intramolecular-Excimer Fluorescence Derivatization, Osama Y. Al-Dirbashi,1 Minnie Jacob,1 Zuhair Al-Hassnan,2 Fahad El-Badaoui,1 and Mohamed S, Rashed1,2* (Departments of 1Genetics and 2Medical Genetics, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia; * address correspondence to this author at: Department of Genetics, King Faisal Specialist Hospital and Research Center, PO Box 3354, Riyadh 11211, Saudi Arabia; fax 966-1-442-4546, e-mail rashed@kfshrc.edu.sa)

Methylmalonic acidemias, a group of heterogeneous disorders, are characterized by accumulation of methylmalonic acid (MMA) and its byproducts in biological fluids (1, 2). Methylmalonic acidemia is now included in all tandem mass spectrometry (MS/MS)-based newborn screening programs (3–5). Detection is based on the finding of increased propionylcarnitine and/or increased propionylcarnitine-to-acetylarnitine ratio in dried blood spots (DBS) by MS/MS. These markers, however, are not specific because they are increased in propionic acidemia and, possibly, in multiple carboxylase deficiency (3). In most programs, newborns or patients with initial positive results are recalled for a second blood spot, and a urine sample is collected for organic acid analysis to differentiate among the three disorders.

In the present study, we used the intramolecular-excimer fluorescence derivatization approach of Nohta and coworkers (6, 7) to form a fluorescent derivative of MMA. This would allow the detection of MMA in DBS samples from affected neonates, leading to a conclusive diagnosis with the remains of the DBS within a short time, often the same working day.

From a DBS, four 3.2-mm discs were punched and extracted into 250 μL of methanol containing 20 μmol/L malonic acid (MA) as internal calibrator by vortex-mixing for 30 s and standing at room temperature for 1 h. After evaporation and reconstitution of the residue in 50 μL of water, we successively added 25 μL of 0.5 mol/L 1-ethyl3-(3-dimethylaminopropyl)carbodiimide (Sigma) in water, 25 μL of 400 g/L pyridine in dimethylsulfoxide, and 50 μL of 15 mmol/L 1-pyrenebutyric hydrazide (Fluka Chemie) in dimethylsulfoxide, tightly capped and vortex-mixed the vials, and left them to stand at room temperature for 5 h. The injection volume was 5 μL, but injections up to 20 μL were possible without adverse effects on the resolution. Moreover, in case of a limited DBS sample, two punches can be used with the proportionate reduction in reagent volumes described above.

The dilabeling of MMA was confirmed by MS analysis (Quattro micro API; Micromass) with purified derivatives obtained by fractional collection. Two abundant ions at m/z 687 and m/z 709 appeared in the spectrum, which correspond to [M+H]$^+$ of pyrene-dilabeled MMA derivative and the [M+Na]$^+$ adduct, respectively.

Chromatography was performed on a Waters Breeze HPLC System, a model 2475 multi λ fluorescence detector (Waters) and a C8 Symmetry column [3.9 × 150 mm (i.d.),