Although mutagenic primers are used in this method, it is important to note that they are equally mismatched to both the wild-type and mutant alleles. The specificity is introduced by extension of the primers, which introduces a different base at the first step. This is an important feature of the assay design, removing any possibility of allele-specific amplification, and produces a robust procedure.

This technique provides an improved method of detection for both the C282Y and H63D mutations and diagnosis of hemochromatosis. By testing simultaneously for both mutations, this method decreases both the cost and time involved for each assay. The PCR is rapid and yields a consistently high-quality product. This assay is appropriate as a diagnostic test because results can be obtained quickly and confidently at a low cost, making it suitable for introduction into routine clinical laboratories.

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References


Development and Multisite Evaluation of an Automated Assay for B12 on the Abbott AxSYM Analyzer, David H. Wilson, Janet Yu, Arden Karian, Janet Kozlowski, and Sara O’Reilly (1) Department of Thyroid, Metabolic, and Cardiovascular Diagnostics R/D and (2) Metabolic Business Team, Abbott Laboratories, Abbott Park, IL 60064; author for correspondence: fax 847-938-7920

Reliable measurement of serum B12 is important in the differential diagnosis of megaloblastic anemia (1). Numerous assays, many of which are manual isotopic methods, have been developed for quantifying B12. The shortcomings among the more recently developed automated methods include a need for frequent calibration, a lack of random access capability, complicated reagent preparations, and slow turnaround time. We have developed an automated, nonsotopic assay for measuring B12 in human plasma and serum with the Abbott AxSYM random access analyzer (2) that improves on existing B12 assays.

During the assay, sample is first mixed with denaturant and extractant reagents. The denaturant reagent contains NaOH to unfold endogenous B12-binding proteins to release B12 for assay. These endogenous binding proteins include intrinsic factor (IF), transcobalamin-II (TC-II), and R-protein. IF exhibits almost no affinity for physiologically inactive forms of B12 (including cobinamide), whereas TC-II and R-protein bind both physiologically inactive B12 and physiologically active forms (e.g., cyanocobalamin). The extractant reagents contain an excess of cobinamide and cyanide. The cobinamide occupies TC-II and R-protein binding sites to facilitate extraction of the B12, and the cyanide converts the unstable physiological forms of B12 (methylcobalamin, hydroxycobalamin, and adenosylcobalamin) into the stable cyanocobalamin form. After the extraction step, microparticles coated with purified hog IF are added and allowed to incubate for 30 min. This step neutralizes the reaction mixture and captures the cyanocobalamin onto the microparticles. The sample/microparticle mixture is then transferred to the AxSYM Matrix Cell where the microparticles bind irreversibly to the glass fiber matrix. After a wash step, a conjugate of B12 hexylamine (a B12 analog) and alkaline phosphatase is added to probe for free IF binding sites on the matrix. After a second wash, methylumbelliferyl phosphate is added and the rate of dephosphorylation by bound alkaline phosphatase is measured. The rate of signal development is inversely proportional to the amount of B12 in the specimen.

Calibrators with spectrophotometrically assigned cyanocobalamin concentrations from 0 to 2000 ng/L were prepared in a base of human serum with B12 removed by charcoal treatment. The calibrators provided with the AxSYM B12 kit were prepared in a base of buffered human serum albumin. Recovery studies indicated no significant difference between these two matrices.

Sample processing time is ~34 min. Throughput in the batch mode is 53 tests/h, and with mixed load lists including folate and ferritin assays, throughput is ~40 tests/h. B12 concentrations are calculated by the AxSYM
by use of a stored calibration curve. Depending on user preference, the assay can be calibrated using either a Master Calibration (two-point) or a Standard Calibration (six-point) procedure. The Master Calibration procedure utilizes a bar-coded Master Curve for each lot of reagents. After the bar-code information is entered into the AxSYM system, the assay can be calibrated by the two AxSYM B₁₂ Master Calibrators. Alternatively, the Standard Calibration procedure does not require the bar-coded Master Calibration curve information, but utilizes the standard set of six AxSYM B₁₂ calibrators. The assay calibration is ordinarily stable for at least 3 weeks.

Across 51 determinations and three reagent lots, the analytical detection limit (zero dose plus 2 SD) of the assay ranged from 7.0 to 50.7 ng/L, with a mean of 25.7 ng/L. Potential interfering substances were tested by assaying serum samples to which test substances were added. There was no interference from bilirubin (200 mg/L), hemoglobin (10 g/L), or lipids (10 g/L). R-protein did not interfere in the assay at 1000 ng/L B₁₂-binding capacity units. Cross-reactivity with cobinamide was <0.0001%. Analytical recovery of 1000 and 1500 ng/L B₁₂ added to 10 serum specimens averaged 104%.

Dilution linearity studies were conducted at six clinical sites. At each site, three specimens with B₁₂ values measured in the upper half of the assay range were diluted serially with the AxSYM B₁₂ Calibrator A (0 ng/L B₁₂). The mean results across four serial dilutions ranged from 91% to 132% of the undiluted value, with a grand mean recovery across all 18 specimens and dilutions of 108.1%.

Precision was evaluated in four different laboratories using NCCLS Protocol EP5-T2. Each of the AxSYM B₁₂ Controls (Low, Medium, and High) was assayed using the AxSYM B₁₂ assay in replicates of two, at each of two separate time points per day, for 20 days, using a single lot of reagents and a single calibration per instrument. Total CVs (3) obtained across all analytical runs and laboratories were 9.7% (range, 8.9–10.5%), 8.3% (range, 7.1–9.3%), and 8.5 (range, 6.7–9.9%) at mean control concentrations of 195, 417, and 855 ng/L, respectively.

Table 1. Method comparisons.

<table>
<thead>
<tr>
<th>Methods*</th>
<th>Lab</th>
<th>n</th>
<th>Slope Intercept</th>
<th>r</th>
<th>S_{xy}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 vs 2</td>
<td>1–6</td>
<td>1993</td>
<td>0.92 ± 0.01</td>
<td>11.4 ± 6.4</td>
<td>0.97</td>
</tr>
<tr>
<td>1 vs 3</td>
<td>1, 3, and 6</td>
<td>932</td>
<td>1.05 ± 0.02</td>
<td>−35.2 ± 12.9</td>
<td>0.95</td>
</tr>
<tr>
<td>1 vs 4</td>
<td>4</td>
<td>321</td>
<td>1.07 ± 0.03</td>
<td>−19.2 ± 16.9</td>
<td>0.97</td>
</tr>
</tbody>
</table>

* Linear regression analysis of singleton measurements pooled across the indicated laboratories using the indicated assay methods. Method 1, AxSYM B₁₂ (γ-axis); method 2, IMx B₁₂; method 3, Quantaphase B₁₂; method 4, ACS:180 B₁₂. Lab 1, University of Tennessee; lab 2, Medical College of Wisconsin; lab 3, University of Nebraska Medical Center; lab 4, Southwest Washington Medical Center; lab 5, Washington University; lab 6, University of Maryland Medical Center.

Method comparison studies were conducted at six clinical sites between the AxSYM B₁₂ assay and the following three other methods: IMx B₁₂ (Abbott Laboratories), Quantaphase B₁₂ (Bio-Rad Laboratories), and ACS:180 B₁₂ (Chiron). Three AxSYM reagent lots were used in the studies. Pooled results from the studies are given in Table 1. The correlation statistics obtained from individual AxSYM reagent lots and laboratories were consistent with those of the pooled results shown in Table 1.

We estimated percentile intervals for both apparently healthy and B₁₂-deficient populations. Samples from 619 apparently healthy individuals obtained at Abbott Laboratories and four clinical sites gave a mean AxSYM B₁₂ concentration of 474 ng/L, with a range of 100-2437 ng/L. The reference interval, estimated as the central 95% of this population, was 157-1059 ng/L. A population of 108 samples deficient in B₁₂ (as determined by commercially available methods) assayed by Abbott Laboratories and four clinical sites gave AxSYM B₁₂ concentrations ranging from 0 to 194 ng/L. The estimated 97.5 percentile of this population was 187 pg/L. Because results may vary among populations, each laboratory should perform a reference range study with a representative sample population and appropriate statistical methods.

Split-sample comparisons between types of collection tubes for serum/plasma B₁₂ showed no significant differences between serum (including serum separator tubes) and tripotassium EDTA (n = 40) or potassium oxalate (n = 40).

In conclusion, our studies indicate that the AxSYM B₁₂ assay is sensitive and precise, and its results correlate well with those of a manual isotopic method, as well as two automated methods. With true random access capability, 3-week calibration stability, quick turnaround time, and no user-reagent preparation, the AxSYM B₁₂ should be an attractive method for assessing B₁₂.

References