RBP-EIA:
A New Approach to Assessing Vitamin A Deficiency
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PATH champions the development, introduction, and adaptation of appropriate and innovative solutions to public health challenges.

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RBP-EIA: A New Approach to Assessing Vitamin A Deficiency

This guide has been developed to introduce the retinol binding protein enzyme immunoassay (RBP-EIA) to potential advocates and users, and to provide information about its prospective role in public health. The guide is organized into the following sections:

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At three landmark conferences—the 1990 World Summit for Children in New York, the 1991 Policy Conference on Ending Hidden Hunger in Montreal, and the 1992 International Conference on Nutrition in Rome—world experts on child nutrition and health ministers committed to work toward the elimination of vitamin A deficiency (VAD) and its consequences by the year 2000. In response to these events, the United States Agency for International Development (USAID) Office of Health and Nutrition under the Bureau for Global Health invested in a set of activities that would change the public health horizon in developing countries for the coming decades.

USAID invested in research that provided evidence of the link between vitamin A supplementation and child survival, and played a key role in establishing the International Vitamin A Consultative Group (IVACG). IVACG has since been instrumental in setting global policies by bringing donors, policy makers, and country-level health managers together on a regular basis to promote methods for assessing VAD and identifying interventions to control its devastating consequences, especially among preschool children (ages 6 to 59 months).

As a result of these and other sustained efforts, VAD has been recognized as a significant public health problem, and countries are now taking steps to implement VAD control programs. Constraints facing the rapid assessment of VAD, however, have been encountered and recognized on both national and global levels. The most significant challenge is a lack of affordable, valid, and reliable assessment tools appropriate for low-resource settings.

As part of the global effort to eliminate VAD, the Program for Appropriate Technology in Health (PATH), under the HealthTech program, received funding from USAID’s Office of Health and Nutrition to develop the retinol binding protein enzyme immunoassay (RBP-EIA). This test was developed to evaluate the prevalence of VAD on a population level.
Introduction

Goals

The RBP-EIA represents an approach to overcoming the constraints that impact the rapid assessment of VAD. The PATH team expects that the RBP-EIA will enhance the reliability and ease of VAD assessment, as well as decrease the associated cost.

More specifically, PATH expects that this new approach will:

- increase the number of prevalence surveys conducted to assess VAD;
- facilitate the monitoring of interventions by tracking changes in vitamin A status;
- improve the consistency of the results of vitamin A assessment, including:
  - ease of specimen analysis and interpretation;
  - reliability of VAD estimates; and
- increase the knowledge base for recognition of VAD worldwide, which ultimately will affect policy and program development.

Furthermore, PATH believes that the RBP-EIA will contribute to long-term efforts to reduce VAD by providing accurate information that will:

- help generate policy guidelines for well-targeted vitamin A programs;
- encourage national commitment of resources to vitamin A programs;
- leverage resources and investments for vitamin A programs;
- inform the design and planning of national vitamin A control programs; and
- be applied to public health research findings and lessons learned.
Despite the difficulty of estimating the global magnitude of micronutrient malnutrition, there is sufficient information to approximate the number of people affected. The most recent estimates indicate that, worldwide, up to 3.5 billion people are affected with micronutrient malnutrition. Of these, more than 100 million are children affected by VAD.

The estimated prevalence of micronutrient deficiencies varies by region. At least half of the world’s burden of micronutrient malnutrition occurs in South Asia, where half of all childhood and maternal deaths occur. In sub-Saharan Africa, VAD is widespread: estimates of VAD (serum retinol < 0.70 µmol/L) in children range from approximately 25% in the Greater Horn region to 55% in some West African countries. While the overall prevalence of micronutrient deficiencies is lower in Latin America, some countries exhibit relatively high prevalence rates of VAD.

The Challenge of Measuring VAD

In the early 1920s, researchers recognized the link between blindness and VAD. They also observed increased morbidity and mortality among adults and children suffering from night blindness. Their findings indicated that supplementing vitamin A intake could be a feasible public health intervention for reducing morbidity and mortality in micronutrient-deficient populations.

In the 1970s, researchers studying blindness caused by VAD noted reductions in child mortality in areas where vitamin A had been administered, even among children who did not have overt clinical manifestations of VAD. It was not until the late 1980s and early 1990s, however, that researchers documented the link between depressed vitamin A status (as indicated by low serum retinol) and an increased severity of morbidity and mortality. The resulting realizations are significant: First, children with marginal vitamin A status face a significant risk of mortality. Second, for every child with symptoms of clinical VAD, there may be several times as many with milder stages of VAD.
While many individuals with clinical VAD have retinol levels below 0.70 µmol/L and show clinical symptoms such as night blindness or xerophthalmia, individuals with marginal or moderate vitamin A status do not necessarily present clinical manifestations.

To establish the prevalence of VAD, researchers and public health officials collect and assess biological specimens. If biological specimens are not available, or if the vitamin A levels cannot be accurately ascertained from the specimens, researchers rely on dietary intake data or the prevalence of clinical VAD estimated from clinical symptoms. Once VAD-control programs begin to take effect, VAD declines dramatically, making it essential to monitor vitamin A status and assess progress towards the elimination of VAD.

Regardless of the degree of VAD, the link between its prevalence and increased morbidity and mortality substantiates the need to improve vitamin A intake in at-risk populations. VAD-control programs offering supplementation, fortification, or dietary change are instrumental in addressing this need.

### Serum Retinol: An Accepted Biological Indicator

Currently, serum retinol is the most widely accepted biological indicator for measuring vitamin A status, and analysis by high-performance liquid chromatography (HPLC) is the traditional reference or “gold standard” method for quantifying serum retinol.

The analysis of retinol by HPLC requires a relatively large sample volume and centralized laboratory facilities with skilled laboratory staff. Specimens are typically taken from populations in the field and then transported to a central facility for storage and laboratory analysis. In some instances, the specimens are exported to another country for analysis.

This process may take several months to complete. The special handling and transportation requirements can dramatically increase the cost of the VAD assessment. These procedures also increase the risk that the samples may become damaged, which would reduce the integrity of the data.
These challenges have prompted researchers to explore the development of a rapid, inexpensive, and quantitative tool for determining vitamin A status at a population level. Retinol binding protein (RBP) has recently been proposed as a surrogate marker for retinol because of the approximate 1:1 molar correlation in serum between retinol and RBP. RBP also offers certain advantages. For example, RBP is a serum protein and resistant to environmental conditions, increasing the likelihood that a relatively simple, quantitative immunoassay could be developed.

Key Characteristics of RBP

RBP has been identified as an ideal analyte to estimate serum retinol, the traditional biological marker for VAD. Because of its robust chemical structure as a serum protein and its 1:1 molar relationship to serum retinol, RBP is an appropriate molecule to use as a surrogate marker for retinol.

RBP has a molecular weight of 19,000 to 21,230 Daltons, while retinol has a molecular weight of 286.5 Daltons. The function of RBP is to bind to and transport retinol from its point of synthesis in the liver to the specific receptors of cells requiring vitamin A.

Two forms of RBP—holo-RBP and apo-RBP—have been described, and correspond to RBP that is liganded and unliganded to retinol, respectively. When a molecule of RBP is synthesized in a hepatocyte, it rapidly binds a molecule of retinol as it transits the endoplasmic reticulum. The synthesis of RBP and its secretion from hepatocytes is controlled by retinol. When the newly synthesized RBP has bound to a retinol molecule, it is considered holo-RBP. The holo-RBP is released into the bloodstream.

Generally, retinol holo-RBP circulates in the plasma bound to another, larger protein molecule called trans-thyretin, or TTR (also known as pre-albumin). Researchers believe that the formation of this retinol-RBP-trans-thyretin complex prevents the loss of the relatively small RBP molecule by filtration through the renal glomeruli and also further stabilizes the binding of retinol to RBP.

Recognition of retinol-RBP-trans-thyretin by a cell surface receptor causes the RBP to release the retinol to the cell. In doing so, apo-RBP is formed, and its associated conformational change drastically reduces its affinity for trans-thyretin. The apo-RBP molecule is then excreted through the kidney glomerulus, reabsorbed in the proximal tubule, and degraded. Hence, each RBP molecule transports only a single retinol molecule before it is degraded.
The Immunoassay

With USAID funding, PATH has developed and validated a rapid, semi-quantitative enzyme immunoassay (EIA) method for detection and quantification of RBP in serum as a surrogate marker for retinol. The RBP-EIA, which has been developed as a kit, is simple to use, requires a small amount of specimen, and takes less than 40 minutes to perform.

Compared to other VAD-detection methods, the RBP-EIA will:
• reduce reliance on centralized laboratory facilities in developing countries;
• save time and money;
• preserve the integrity of samples by eliminating the need to transport specimens to a second location for analysis by sophisticated and expensive methods such as HPLC;
• provide a more cost-effective tool for monitoring and recognizing VAD;
• reduce the time between initial assessment and availability of results.

Thus the RBP-EIA provides an opportunity to overcome the significant training, cost, and time required for performing HPLC analysis. Once the RBP-EIA is commercially available, it will provide health care managers with a rapid, inexpensive, and effective tool for assessing the extent of VAD within a population. To further simplify VAD assessment, future RBP-EIA refinements will allow for the analysis of dried blood spots collected by finger prick.

PATH expects that the RBP-EIA will facilitate national vitamin A assessments and increase the number of countries that will be able to conduct VAD prevalence surveys, especially in areas where the surveys have not yet been performed.

References


As discussed in the previous section, the special equipment, training, and handling requirements of HPLC—the reference method for analysis of retinol—are not feasible for most low-resource settings.

Researchers have recognized that a rapid, inexpensive, and semi-quantitative tool is needed to determine vitamin A status at the population level to meet certain objectives. RBP has recently been demonstrated to be a surrogate marker for serum retinol and therefore may serve as an alternative marker for assessing vitamin A levels in low-resource settings.

**Specifications of Existing Tests**

Several methods, devices, or kits capable of assessing serum retinol or RBP have been developed or are under development.

Table 2.1 summarizes the types of specimens, equipment, time, and costs of each test, as well as their performance. More complete information is available through the references cited at the end of this section.
### Table 2.1 Methods and Kits for Assessing Vitamin A Deficiency

<table>
<thead>
<tr>
<th>Technique</th>
<th>Developer</th>
<th>Analyte Detected</th>
<th>Specimen</th>
<th>Equipment Required</th>
<th>Time Required</th>
<th>Cost</th>
<th>Performance (Sens/Spec)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tests in Development or Research</strong></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>RBP-EIA</td>
<td>PATH</td>
<td>RBP</td>
<td>Serum</td>
<td>Plate or strip well reader, washer, micropipettes</td>
<td>35-40 minutes per 48 samples; average 400 samples per day</td>
<td>Commercial product not yet available. Cost estimate is US$3.00-$5.00 per determination</td>
<td>Sensitivity: 71% Specificity: 92%</td>
</tr>
<tr>
<td>Futterman Fluorescence Assay</td>
<td>Centers for Disease Control and Prevention</td>
<td>Retinol</td>
<td>Serum, Plasma</td>
<td>Fluorimeter or scanning spectrophotometer, micropipettes</td>
<td>20-40 samples per day</td>
<td>No data available</td>
<td>Method has not been validated for this application</td>
</tr>
<tr>
<td>Radio-Immunooassay (RIA)</td>
<td>Columbia University</td>
<td>RBP</td>
<td>Serum, Plasma</td>
<td>Isotope, micropipettes, vortex mixer, emission counter</td>
<td>No data available</td>
<td>Research method; not commercially available</td>
<td>Sensitivity: 87% Specificity: 98%</td>
</tr>
<tr>
<td><strong>Commercially Available Tests</strong></td>
<td></td>
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</tr>
<tr>
<td>RBP-EIA</td>
<td>Immunodiagnostik</td>
<td>RBP</td>
<td>Serum, Plasma, Urine</td>
<td>Plate or strip well reader, washer, incubator, micropipettes</td>
<td>12 hours per 48-96 samples</td>
<td>US$9.00-$18.75 per determination</td>
<td>Research assay; performance not documented</td>
</tr>
<tr>
<td>Radial Immunodiffusion Assay (RID)</td>
<td>The Binding Site</td>
<td>RBP</td>
<td>Serum, Plasma</td>
<td>Micropipettes, jeweler’s loupe, passive light source</td>
<td>96 hours per 42 samples</td>
<td>US$4.17 per determination</td>
<td>Not documented</td>
</tr>
<tr>
<td>HPLC</td>
<td>Bio-Rad Craft Technologies</td>
<td>Retinol</td>
<td>Dried blood spot, Serum, Plasma, Urine, Breast milk</td>
<td>HPLC system, centrifuge, micropipettes, vortex mixer</td>
<td>20-25 samples per day</td>
<td>US$10.00-$25.00 per determination</td>
<td>Reference method; considered gold standard</td>
</tr>
</tbody>
</table>
**Immunodiagnostik** (Bensheim, Germany) produces an EIA for RBP in a microtest plate format. This test involves a significant amount of preparation and is therefore labor intensive, relatively difficult to use, and has a low sample throughput. The prime drawbacks are that it is a “research” assay and that the kit is expensive. Each 96-test kit costs US$900, or more than US $9 per sample.

**The Binding Site** (San Diego, CA) uses a radial immunodiffusion assay (RID) method for their RBP test. The RID is a relatively simple method, but takes up to 96 hours to complete, and the results often vary due to subjective reading of the test reactions using a jeweler’s loupe. The small sample volume (5 µl) per test can be a disadvantage, as it is difficult to accurately and repeatedly pipette this volume into the test wells. This assay costs approximately US$4 per determination.

**The Centers for Disease Control and Prevention** (Atlanta, GA) has developed a test to determine serum retinol by fluorometry, called the Futterman fluorescence method. This is a fairly simple analytical method that requires a relatively large specimen volume. Fluorometry has not been fully validated as a method for determining vitamin A status. The fluorescence method may measure several undefined analytes in addition to the RBP-retinol complex, which may compromise test specificity. (Also see Futterman et al., 1975.3)

**Craft Technologies** (Wilson, NC) has developed instruments that measure retinol using serum and serum eluted from dried blood spots. Although their work using dried blood spots as samples is promising, the test requires the use of HPLC and the price per test is expensive (US $15 to $20 per determination). All testing currently needs to be performed at the Craft Technologies laboratory, which may limit the test’s suitability for field application. (Also see Craft, 2001.4)

**Bio-Rad** (Hercules, CA) manufactures an HPLC kit for determination of vitamin A/E. This kit includes all reagents needed to perform 100 to 150 retinol determinations. The kit is easy to use and the results are highly reproducible, since all reagents and components are manufactured under Good Manufacturing Practices (GMP) conditions. Samples can be prepared in minutes. Unfortunately, this kit is relatively expensive, and is not marketed in the United States because it is not approved by the Food and Drug Administration (FDA). If this test kit were adopted by VAD assessment teams as a reference method, variation between methods would be significantly reduced, and results would be more accurate and dependable than existing retinol HPLC methods.
Columbia University (New York City, NY) has developed the radio-immunoassay (RIA) to determine RBP status. This method is very sensitive and specific, but is only a research tool at this time. It is not commercially available, and would be impractical for routine field use since it uses radioactively labeled antibodies and requires a specialized detector to measure signal. (Also see Blaner, 1990.4)

Notes and References

1. For general information on indicators for assessing VAD, see the World Health Organization’s *Indicators for Assessing Vitamin A Deficiency and Their Application in Monitoring and Evaluating Intervention Programmes* (WHO/NUT/ 96.10, Geneva, 1996).


Program planners working at local, national, or regional levels often want to measure vitamin A status in populations and estimate the prevalence of deficiency. Their efforts may stem from interest in establishing baseline figures of VAD prior to launching an intervention, tracking progress in control efforts, or identifying risk factors associated with VAD. Whatever the reason, the specific goal must be clearly identified prior to initiating the VAD assessment.

Regardless of the indicator used to quantify vitamin A status, the appropriate study design and sampling strategy depend on the primary reason for the VAD assessment. It is important to set out clear objectives for the study by explicitly specifying the questions that need to be answered, the population of interest (e.g., children ages 6 to 59 months or women of childbearing age), groups in need of the information, and the manner in which the information will be used.

Numerous other factors—including the available resources, cost of conducting the study, the desired precision and confidence in the estimates, and the timeliness of the results—will influence the study design and sampling strategy used.

The RBP-EIA is a competitive enzyme immunoassay that detects and quantifies RBP in human serum. The test uses purified human RBP adsorbed to microtest strip wells to compete with natural RBP found in serum.

**Test Characteristics**

Prior to developing the RBP-EIA, PATH identified the characteristics that a test must have to improve researchers’ ability to assess VAD on a population level. PATH researchers then used these characteristics to guide the development of the RBP-EIA.

PATH concluded that an ideal test would be:
- quantifiable;
- rapid;
- simple;
- easy to perform;
Section 3

- able to provide high throughput compared to conventional methods;
- able to generate population estimates of VAD that correlate with those from retinol;
- volume-efficient, requiring only a small sample (10 µl per determination);
- cost-efficient;
- able to be performed independently of a reference or sophisticated laboratory;
- robust;
- reproducible.

The RBP-EIA fulfills each of these criteria.

The Technical Platform for the RBP-EIA

To develop the RBP-EIA, PATH selected the monoclonal antibody (MAb) clone 8 from a panel supplied by the University of Massachusetts, which was then licensed. PATH developed a competitive EIA format in a microwell format using the single MAb. This system is less complex than conventional capture-signal systems that use two MAbs or a MAb and polyclonal sera that may involve a third antibody reagent conjugated to an enzyme.

The single-MAb approach has several advantages, including a shorter assay time (35 to 40 minutes), the need for only a single wash step, and a quantitative result in the key range of 10 to 40 µg RBP/ml (0.48 to 1.92 µmol RBP/L).

The competitive EIA detects RBP in serum by comparing the optical density (OD) reading of each sample with the OD of normal-, moderate-, and low-calibrator sera included in the kit. The assay results are linear within this region. Like most competitive assays, sera producing weaker reactions (lower ODs) represent normal vitamin A levels, while stronger reactions (higher ODs) represent VAD.

In this document, when referring to PATH’s RBP test, units will be expressed as µg RBP/ml. When comparisons are made, 10 retinol units of RBP will be expressed as µmol RBP/L.

To perform the assay, the specimens and calibrator sera are diluted in assay buffer and added to the individual wells. A monoclonal, anti-RBP antibody, conjugated to horseradish peroxidase (HRP) enzyme, is diluted and then immediately added. The test is incubated at room temperature for 15 minutes and then washed.
Tetramethylbenzidine (TMB) enzyme substrate is added, incubated for 10 minutes, and stopped with acid. The test is immediately read with a plate reader, and the results are calculated based on values obtained from the calibrator sera. The test results are available 35 to 40 minutes after the start of the assay. For highest accuracy, all samples, including calibrators, should be run in duplicate. Including controls and samples, each assay test plate provides 48 results.

The product insert in Appendix B provides detailed instructions for obtaining appropriate specimens, performing the test, and determining the results. Section 6 addresses troubleshooting problems that may arise in the laboratory.

The RBP-EIA is designed to assess and monitor the VAD status in populations. While the results of the assay are quantitative, they should not be considered to be diagnostic of VAD for individuals. Rather, they should be used as a research tool to assess vitamin A status and the extent of VAD in populations.

Performing the Test

A detailed description of preparing and performing the test can be found in Appendix B.

An overview of the test process can be found in figure 3.1.
**Figure 3.1 Overview of the RPB-EIA Test Process**

1. Dilute samples 1:25 = 240 µl sample diluent 10 µl patient sample
2. Add 100 µl of diluted patient sample to test wells
3. Dilute conjugate
4. Add 100 µl of diluted conjugate to each test well
5. Incubate 15 minutes at room temperature (RT)
6. Wash 5 times
7. Add 200 µl of substrate per test well
8. Incubate 10 minutes RT
9. Add 100 µl of stop solution per test well
10. Read $A_{450}$
The following sections are intended to substantiate the technology used to develop the RBP-EIA, describe particular technical considerations that apply to the test use, and address technical questions about the test and its performance.

**Using the RBP-EIA**

**Q** Is the RBP-EIA sufficiently fieldfriendly and appropriate for use in developing-country clinics and laboratories?

**A** Yes, the RBP-EIA is appropriate for use in the field and/or low-resource settings, provided that laboratory equipment is in good working order and supplies are available.

**Q** What are the minimum required laboratory supplies needed to execute the assay?

**A** To perform the RBP-EIA, laboratories must have the following equipment: EIA plate or strip-well reader fitted with a 450-nm filter, EIA plate or strip-well washer, micropipetters and disposable tips, test tubes, a timepiece or laboratory timer, a refrigerator (2° to 8° C), and paper towels or similar absorbent material.

**Q** Who should use the assay?

**A** The RBP-EIA can be performed by laboratory staff who have basic knowledge of pipetting techniques and EIA procedures. Some proficiency training may be needed to ensure that the technical staff are able to run the test. Public health workers, epidemiologists, nutritionists, and other health care professionals interested in a population’s VAD status can interpret the data and apply the results.

**Q** Where can the assay be used?

**A** The RBP-EIA has been developed so that it can be used in any setting where appropriate and well-maintained EIA test equipment is available.
Section 3

Q What are the key elements of training and quality assurance/quality control for standardizing use of the assay in national programs?

A The proper employment of good laboratory practices, proper pipetting techniques, and calibrated equipment are essential to ensuring that the results obtained from the RBP-EIA are reliable.

Q How can an internationally accepted cut-off be established for serum RBP that relates to the present accepted serum retinol cut-off—0.70 µmol/L?

A This cut-off has been established in the literature, and is logical, as retinol and RBP exist in approximately a 1:1 molar ratio.¹

The Assay

Q What is the correlation between the RBP-EIA and retinol levels obtained by HPLC?

A Figure 3.2 compares the RBP-EIA with retinol-HPLC values from a sub-sample of sera from Nicaraguan children. The figure indicates that there is a significant association between the two analytes, with a correlation coefficient of 0.82. Information on additional correspondence analyses is presented in Section 5.¹

Figure 3.2 Correlation Between RBP-EIA and Retinol HPLC

\[ y = 0.6216x + 0.3233 \]

\[ R^2 = 0.8201 \]
Q Does the relationship between serum retinol and RBP hold at extremes of vitamin A status? If not, how critical is this?

A The RBP-EIA was engineered as a population screening tool to provide optimum results between 0.48 and 1.92 µmol RBP/L, which encompasses the critical range in determining vitamin A status in populations.

Q Can the RBP-EIA distinguish between lowered blood levels of RBP due to VAD versus chronic infection? Do retinol measurements reflect true VAD, since inflammation can also result in reduced retinol levels?

A PATH is not aware of any test methods currently capable of distinguishing between lowered retinol and RBP levels due to nutritional deficiency or inflammation. If inflammation is a concern in a population with apparent VAD, questionnaires or clinical examinations should be administered to subjects along with testing of acute phase proteins to determine the contribution of acute or chronic inflammation.

Q What are the key parameters of assay performance relative to serum retinol?

A The RBP-EIA’s key assay performance parameters contrast in several ways with the analysis of retinol by HPLC:

- Sample preparation for the EIA relies on dilution, whereas the HPLC method incorporates extraction and dilution methods, which can introduce additional sources of error.
- The RBP-EIA is self-contained and performed with supplied reagents, while HPLC, with its need for column quality and integrity, instrument pressure, and injection consistency, requires many extraneous factors for optimal performance.
- The EIA is more resource friendly than current retinol quantification methods with respect to cost, time, reproducibility, and ease of use.
- The throughput of the RBP-EIA is greater than that of the retinol-HPLC method because 48 samples can be simultaneously assayed in significantly less time than it would take to obtain the same number of results by HPLC.

Q What are the characteristics of the monoclonal antibody (MAb) selected for use in the RBP-EIA?

A For the development of the RBP-EIA, PATH screened a number of anti-RBP MAbs and an anti-RBP polyclonal serum. These were obtained from commercial sources.
and the University of Massachusetts. The antibodies were evaluated for use in a quantitative assay as well as coupling with HRP for use as signal. PATH selected clone 8 (43-D2) from the University of Massachusetts, an IgG antibody, as the best candidate, and designated another MAb with equivalent performance as a backup.

Initial feasibility experiments with clone 8 included multiple dilutions of antiserum and reference RBP antigen to determine relative sensitivity of the system. The sensitivity limit of an immunochromatographic strip format was found to be <9 mg RBP/ml under optimal conditions—values that are approximately a thousand-fold lower than normal levels of RBP found in sera. The selected MAb does not react with human C-reactive protein, rheumatoid factor, bilirubin, hemoglobin, intact red blood cells, triglycerides (as triolein), L-thyroxine, retinol, estrone acetate, β-estradiol, or trans-thyretin.

Q Does the selected MAb distinguish between holo- and apo-RBP?

A While PATH does not have definitive data regarding the MAb’s ability to distinguish the two forms of RBP equally, we have presumptive data from spike/recovery studies of urinary RBP in human serum. Analyte recovery is equal to or greater than 70%, based on Lowry total protein concentrations provided by Sigma.

Additionally, we have performed inhibition curve studies using RID plate calibrators (characterized as holo-RBP by the manufacturer), and characterized human serum from Behring (apo- plus holo-RBP). The resulting studies produced parallel inhibition curves, indicating that both apo- and holo-RBP are recognized. Compared to both curves, samples were within a 12% average across the dynamic range of the assay and resulted in no mischaracterization of overall VAD status.

Q Is the ability of the MAb to bind to RBP influenced by the concentration of trans-thyretin in the circulation?

A No. During validation studies of the RBP-EIA, excess trans-thyretin was introduced into normal human serum at 800 µg/ml to assess the degree of possible assay interference. The resulting data demonstrated an average test/reference (spiked/control serum) ratio of 1.03. The average inter-assay variability has been determined to be 3.2%, which demonstrates the MAb’s ability to detect RBP independent of the concentration of trans-thyretin.
In severely malnourished populations, however, protein deficiency can occur. Since the trans-thyretin-RBP-retinol complex is interdependent, marginal protein status may influence the concentration of RBP. For this reason, lower levels of RBP would be expected, though not related to the MAb’s ability to bind RBP.

**Q** Does the selected MAb recognize serum and urinary RBP equally well? Would serum or recombinant RBP be better?

**A** In early studies, PATH's research team prepared an affinity column using the MAb. We then purified RBP from normal human plasma by column chromatography. We eluted the resulting RBP and compared it to the urinary RBP from Sigma in the EIA format. We then assessed protein concentrations of both RBPs by standard protein determinations, and dilutions of RBP were then made in depleted human serum. When assayed by the RBP-EIA, the two sources of RBP produced equivalent results, suggesting that the serum RBP reacted no differently than urinary RBP in the assay.

Commercial sources of purified RBP that PATH has used include Sigma, Dade Behring, and The Binding Site. PATH also made an RBP affinity column using the MAb to purify our own RBP from serum, but we were concerned about stability issues. Behring’s protein standard contains several other serum proteins in addition to RBP, which makes it unsuitable as a competitor for the solid phase of the assay. The RBP from The Binding Site was supplied as purified holo-RBP, but was cost prohibitive and therefore not used for assay development. The Sigma RBP is purified from urine from patients with proteinuria. Although there may be some RBP degradation in the Sigma material, it is still highly reactive with our MAb, and conformational modifications have not been a significant factor. Using urinary RBP in a competitive format does not present a problem, since the epitope recognized by the conjugated MAb we are using is intact.

**Samples and Sample Collection**

**Q** Do researchers have to analyze more samples for RBP by EIA to have the same level of precision as retinol?

**A** No. The RBP-EIA is as precise within its calibrated range (both within and between assays) compared to retinol by HPLC methods. Therefore, there is no need to collect or analyze additional samples for the assessment of population VAD.
Q **What percent of samples analyzed for RBP by EIA should be subsampled for retinol by HPLC? For how long?**

A This is a judgment call for both epidemiologists and statisticians who arrange the studies. PATH’s opinion is that assessing retinol by HPLC in 5% to 10% of the total samples is sufficient until the RBP-EIA is more widely accepted. After RBP has gained acceptance as a surrogate marker for VAD, it may no longer be necessary to obtain retinol data to ensure comparability. PATH feels that this “comfort level” will only come with time and as the test is accepted by the vitamin A community.

Q **Should the RBP-EIA be validated for whole blood as well as serum?**

A The RBP-EIA has been validated using serum specimens. PATH’s early results have indicated that plasma is a less satisfactory sample for the RBP-EIA. Whole blood may not be appropriate because intact red cells, certain anticoagulants, and fibrinogen interfere with the EIA. Further research is required to explore the feasibility of using whole blood as a sample.

Evidence, however, indicates that dried blood spots (DBS), subsequently eluted from the filter paper matrix by overnight incubation in assay buffer, appear to be promising samples for use with the RBP-EIA.

Q **What steps are needed to validate the RBP-EIA with DBS specimens?**

A PATH’s preliminary work indicates that DBS specimens may be used for the analysis of RBP using the EIA platform. PATH is currently planning larger-scale experiments on sample collection, storage, and handling conditions. Upon successful completion of this research, we plan to conduct a parallel study in which RBP levels analyzed from serum will be compared with RBP levels in DBS.

Q **Are studies needed to examine the effects of sample collection, handling, storage, and processing on stability of RBP (e.g., effects of time, temperature, and bacterial contamination)?**

A According to principles of good laboratory practice, sample collection, processing, storage, and handling of blood prior to conducting an assay are crucial factors for ensuring accurate results. The proper handling of samples for retinol and RBP has been previously described. PATH has performed small-scale investigations.
of these issues that indicate serum RBP is stable for 8 hours at 2° to 8°C, and for at least six months at -20°C. A larger study may be needed to evaluate these parameters; such research could be incorporated into either a prevalence survey or an evaluation of a micronutrient intervention conducted in a field setting.

**Q** What is known about the stability of RBP in serum samples?

**A** RBP is very stable, even after prolonged periods of exposure at suboptimal storage conditions. It seems only to be degraded by prolonged exposure to extremely high temperatures (greater than 37°C).

**References**


Implementation Issues in VAD Assessments

The following section provides information for program planners and researchers who conduct VAD assessments in the field. It provides information on sample types, sample handling, and necessary laboratory equipment and supplies.

Three types of specimens can be collected and prepared for the assessment of vitamin A status: serum, plasma, and dried blood spots. This section discusses their advantages and disadvantages, along with information about the appropriateness of their use with the RBP-EIA.

Types of Biological Samples

Serum
Traditionally, serum has been the most widely used biological specimen for nutritional assessments. Serum specimens require a delay in processing until a clot has formed, after which the sample is centrifuged to separate the blood clots from the sera. At this time, serum is the only type of biological specimen that has been validated for use with the RBP-EIA.

Plasma
Plasma is relatively easy to collect, as blood can be immediately centrifuged after it is taken, and the plasma component can be easily separated from blood cells. However, during development activities, PATH laboratory staff encountered difficulties establishing a correlation between retinol and RBP using plasma samples. Several experiments have shown that using matched serum and plasma samples in the RBP-EIA introduces a bias into the assay, which varies as the plasma ages and the anticoagulant degrades. This allows the fibrin in the sera to precipitate and nonspecifically trap RBP as well as other serum proteins. Plasma therefore should not be used as a sample in the RBP-EIA.

Dried Blood Spots
The use of DBS would simplify sample collection and allow health care workers to collect more samples per day, since the equipment required for collection is minimal. The use of DBS is easy, and samples are inexpensive to collect and transport. It is

See Appendix C for more information about recent DBS feasibility experiments.
critical that DBS samples are properly collected and completely dried on filter paper cards before storage.

The use of DBS has been investigated in laboratory settings as a potential sample for the RBP-EIA (Appendix C). Results from recent parallel studies demonstrate a good correlation between control serum and DBS collected from the same individuals on the same day. Once PATH has validated the use of DBS for the RBP-EIA, it will likely be the type of specimen recommended for collection.

Sample Handling

Sample handling is a critical element of any study that uses biological specimens to measure vitamin A status. Proper planning and precautionary measures are essential to ensuring the integrity of the specimens. Numerous issues related to collection, handling, processing, transporting, and storing the samples must be considered, including:

- collecting the sample type in sufficient volume;
- using the sample type appropriate for the test method (serum or DBS);
- ensuring that the samples are collected correctly, and that protocols established by the manufacturer for the collection method are followed as described;
- maintaining the cold chain where necessary;
- limiting the time between sample collection and processing (processing refers to the removal of the serum component from the clotted blood cells, and should begin within 2 to 3 hours of collection);
- if a portable centrifuge and power source are available, processing the serum component and separating it from the clotted blood cells at the study site (processing must still occur 2 to 3 hours after collecting the blood samples);
- clearly labeling all samples with unique identifiers;
- avoiding microbial contamination;
- avoiding hemolysis; and
- ensuring that all samples are handled consistently to minimize differences in the samples collected on different days.

Once the serum is separated from the clotted blood cells, the serum samples should be stored consistently. If analysis cannot begin immediately, the serum samples should be stored at 2° to 8°C for up to 24 hours. For extended storage, the serum samples should be frozen at -20°C or lower in a non-self-defrosting freezer. The samples should not be thawed until they can be analyzed by RBP-EIA.
It is of paramount importance to treat the serum samples consistently. For example, if 300 samples are to be collected over a one-month study period, each sample must be handled identically over the entire period, regardless of when the samples are collected or where they are processed.

### Laboratory and Personnel Requirements

The RBP-EIA has been designed to operate with a range of laboratory conditions and equipment. Persons performing the assay must be able to uphold the principles of good laboratory practice, including equipment calibration and proper verification of the purity of reagent-grade water. They must also have the necessary skills and aptitude to carry out the RBP-EIA procedure.

### Equipment Requirements

To perform the RBP-EIA, laboratories must have the following equipment:
- EIA plate or strip-well reader fitted with a 450-nm filter
- EIA plate or strip-well washer
- deionized (DI) water
- vacuum aspirator
- vortex mixer
- micropipettors and disposable tips
- test tubes
- timepiece or laboratory timer
- laboratory markers
- ¼” hole punch (if DBS are used as specimens)
- 2 ml Eppendorf tubes
- refrigerator (2° to 8°C)
- paper towels or similar absorbent material

PATH has evaluated a prototype strip well reader for use with the RBP-EIA as a replacement or substitute for a plate reader; however, further development is required. Once implemented, the suggested design improvements should result in a user-friendly and field-friendly instrument.
Laboratory and Field Evaluation Results

This section summarizes the major findings from PATH’s laboratory experiments and field evaluations of the RBP-EIA. The section provides information on establishing the proof of concept, determining assay performance characteristics and possible interfering substances, and comparing the test to an accepted technology using specimens obtained from the field.

To provide greater technical detail to scientific and academic audiences, PATH is developing additional articles about RBP-EIA validation.

Before techniques such as the RBP-EIA can be widely used for VAD assessment, program planners, researchers, donors, advocates, and potential users must be certain that the technique’s validity and performance characteristics have been rigorously evaluated and that the performance specifications that were previously established have been met. PATH has thoroughly evaluated the RBP-EIA’s performance characteristics as well as its accuracy as compared to retinol-HPLC.

For initial test evaluations, PATH used *The United States Pharmacopoeia and The National Formulary* guidelines to determine how well the RBP-EIA detected the designated analyte, RBP, and if interfering substances inhibited the performance of the test.

Preliminary Studies

In collaboration with the Institute for Nutrition in Central America and Panama (INCAP), PATH conducted development-stage validation of the RBP-EIA in Guatemala. The objective of this study was to assess the performance and practicality of PATH’s RBP-EIA as a surrogate marker for serum retinol, with a panel of reference specimens representing a range of vitamin A status in an initial concordance analysis. In addition, a small study assessed the performance of the RBP-EIA with freshly obtained sera and plasma from volunteers in the local population.

The results of this study indicated that when plasma samples were used, the RBP-EIA did not correlate well with the results of retinol detection by HPLC. This indicated that there was a problem with the use of heparinized plasma specimens. Using plasma as a sample for the RBP-EIA introduced a bias in the quantification of results, leading to higher RBP values in plasma than in serum. The correlation was much closer when comparing the results of RBP-EIA versus retinol detection by HPLC using sera.
Establishing Proof of Concept

Method
To establish proof of concept, PATH tested a panel of blinded sera collected as part of a micronutrient study in Papua, New Guinea. These specimens were obtained from Johns Hopkins University (JHU), who had previously analyzed them for their retinol values. Duplicate samples of sera were tested in PATH’s lab by the RBP-EIA. The results were then averaged, and the data were returned to JHU for comparative analysis.

Results
The results from this panel indicated that within the RBP-EIA’s linear range of 0.48 to 1.92 µmol/L, RBP values correlated well with the retinol levels ($R^2 = 0.84$). These data are presented as Figure 5.1. As indicated by the arrow, the results of all but one specimen correlated well with HPLC retinol. JHU later revealed that retinol had been added to this particular sample for use as a blind internal control to evaluate test specificity.

Figure 5.1. Evaluation of Retinol HPLC Versus RBP-EIA on a Panel of Sera From Papua New Guinea (n=25)
Conclusion
On this limited panel, which spanned the range from retinol sufficiency to deficiency, the results from the RBP-EIA correlated well with retinol-HPLC results generated at JHU. For the calibrated range of the test (0.48 to 1.92 µmol RBP/L), the correlation was very close. The data suggested that the RBP-EIA was close to being optimized, and the current assay formulation could be applied to further evaluations.

Laboratory Evaluation

Analytical Performance Characteristics

Method
The performance characteristics of the RBP-EIA were defined in the PATH laboratory using commercially available reference samples with known RBP concentrations. These could be diluted or used as needed. To determine consistency among test operators, four technicians ran the tests. Each set of results was evaluated and compared to known RBP concentrations to establish the following performance characteristics detailed below.

1. **Accuracy.** Assay accuracy was determined by four technicians who performed the RBP-EIA with samples containing known RBP concentrations, and by comparing their results. These sera were standard dilutions made from purchased calibrators (Dade Behring, Deerfield, IL). The standard curve generated was plotted and used to estimate the RBP content of each sample. The means and standard deviations of each were calculated to determine the accuracy of the test.

2. **Precision.** Assay precision was determined by testing multiple aliquots of samples representing the calibration range of the assay. These data were used to estimate the standard deviation (SD) and coefficient of variation (CV) for each sample and for all samples collectively.

3. **Detection limit.** The detection limit is defined as the least amount of analyte that can be detected by the RBP-EIA, but is not necessarily quantified when the assay is performed according to the prescribed methods. To prepare a test specimen, a normal serum sample was depleted of RBP by affinity chromatography. The sample was repeatedly analyzed by RBP-EIA (n = 24) to obtain true value and zero matrix (buffer only) to determine the means and SD. The signal-to-noise ratio was calculated, and the detection limit was calculated as the difference between the depleted plasma sample value and the RBP value equal to one standard deviation above the mean of the zero matrix.
4. **Quantitation limit.** The quantitation limit is defined as the lowest amount of analyte that can be determined with acceptable accuracy and precision. Multiple samples of buffer with and without RBP were analyzed, and three standard deviations in µg/ml from the mean were calculated from the standard curve.

5. **Linearity.** To determine the linearity of the assay, 23 samples that spanned the assay’s calibration range were tested in duplicate. Samples were diluted 1:2 and 1:4 in assay buffer. The values corrected for dilution were then assessed against the undiluted values to determine dilution accuracy.

6. **Range.** The assay range is determined as the interval between the upper and lower analyte levels that produce suitable levels of precision, accuracy, and linearity. For the RBP-EIA, samples spanning a range of values were analyzed both neat and diluted in assay buffer. The CV of result and linearity were calculated and evaluated for accuracy as defined by both the linearity presented above and reproducibility of the result, not to exceed a 20% CV.

7. **Analyte recovery.** Calibrators in the form of commercial sera were spiked into samples with known RBP values and were analyzed in the RBP-EIA. Analyte recovery was calculated as the test result divided by the expected result.

8. **Intra-assay variability.** The use of 10 replicates of each sample in a single assay determined the intra-assay variability.

**Results**

Due to the large volume of raw data generated, the individual results from each determination are not presented here. The analytical performance characteristics for the RBP-EIA assay are presented below. They include:

1. **Accuracy.** An average of 96% ± 4% of the expected result was obtained within the calibrated range (0.48 to 1.92 µg RBP/L, or 10 to 40 µmol/ml).

2. **Inter-assay precision.** An 8.9% CV was calculated within the calibrated range.

3. **Detection limit.** The assay could distinguish between a blank sample containing no RBP and a sample containing as little analyte as 1.1 µg RBP/ml. While this level of sensitivity is useful for the assay, the assay could be made much more sensitive if needed.
4. **Quantitation limit.** Analytical sensitivity was determined as 7.7 µg RBP/ml.

5. **Linearity.** Linearity was found to be 100% ± 7% in the range of the calibration curve.

6. **Range.** The current assay provided a reliable, linear result between 0.42 and 1.80 µmol RBP/L, for a range of 1.40 µmol RBP. This was very close to the original specification to provide linear results between 0.48 and 1.92 µmol RBP/L. Values of less than 0.42 µmol or greater than 1.80 µmol RBP/L were still reasonably accurate, but produced fewer reproducible results as reflected by higher CVs for RBP. Additional laboratory evidence based on the CV of the 1.92 µmol RBP/L calibrator indicates that the range may be extended from 1.38 to 1.48 µmol RBP.

7. **Analyte recovery.** Recovery from serum averaged 102% ± 11% within the range of the calibration curve.

8. **Intra-assay variability.** A 6.7% CV in the calibrated range was observed.

The analytical performance characteristics for the parameters discussed are summarized in Table 5.1.

**Table 5.1. Analytical Performance Characteristics for the RBP-EIA**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Accuracy</strong></td>
<td>0.96 ± 0.04% in the calibrated range*</td>
</tr>
<tr>
<td><strong>Inter-assay precision</strong></td>
<td>8.9% CV in the calibrated range</td>
</tr>
<tr>
<td><strong>Detection limit</strong></td>
<td>1.1 µg RBP/mL</td>
</tr>
<tr>
<td><strong>Quantitation limit</strong></td>
<td>7.7 µg RBP/mL</td>
</tr>
<tr>
<td><strong>Linearity</strong></td>
<td>0.997 ± 0.07</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>0.42 to 1.80 µmol RBP/L</td>
</tr>
<tr>
<td><strong>Analyte recovery</strong></td>
<td>1.02 ± 0.11</td>
</tr>
<tr>
<td><strong>Intra-assay variability</strong></td>
<td>6.7% CV in the calibrated range</td>
</tr>
</tbody>
</table>

*Calibrated range = 0.48 to 1.92 µmol RBP/L.

**Conclusions**

The analytical performance characteristics of the RBP-EIA have been established and meet the original design specifications for a precise, practical, and dependable test that produces a linear, quantitative relationship between RBP and serum retinol in the critical range of approximately 0.48 to 1.92 µmol RBP/L.
Interfering Substances

The purpose of analytical interference testing is to determine the effect of endogenous and exogenous substances on analytical test results. Ultimately, this information may be used to establish assay limitations and in product labeling claims. PATH conducted interfering substance studies by adding or “spiking” amounts of potential interfering substances into well-characterized normal serum samples with a known RBP concentration and then comparing the resulting RBP concentration of the spiked samples to the normal control samples.

Methods

Interference testing was conducted in accordance with the guidelines in the *The United States Pharmacopoeia and The National Formulary*. The RBP concentration in the sera used for this experiment had been previously characterized. Potential interfering substances that were tested included human serum with increased levels of C-reactive protein (CRP), rheumatoid factor, bilirubin, hemoglobin, human red blood cells, triglycerides (as triolein), L-thyroxine, retinol, estrone acetate, ß-estradiol, and trans-thyretin. These substances and metabolites were tested at a concentration that represented elevated levels above an expected normal range.

Statistical analysis was based on a “paired-difference” approach. A relatively high concentration of the potential interfering substance was spiked into a serum and the RBP value was tested in parallel with its paired normal serum. The difference between spiked and unspiked samples was determined. A dose-response series for each potentially interfering substance was also run if needed to determine whether there was a relationship between the concentration and the interference. If the interfering substance had no effect at high concentration, no further testing was performed.

These tests were intended to reveal whether any common substances interfere with the performance of the RBP-EIA so that labeling claims and limitations could be established. For the substance to be considered “non-interfering” as defined by the USP Methods Validation Study, the bias between the normal and spiked RBP concentration must be less than 12%.

Samples were prepared in the appropriate diluents. Concentrations were prepared by spiking the test material to achieve the desired concentrations in a final volume of 2.0 ml in sera of known RBP concentration (Table 5.2).
Laboratory and Field Evaluation Results

Prior to assay, each sample was thoroughly mixed in triplicate by two analysts in separate assays, and then stored at 4°C until analysis was completed.

To verify that each assay met the validity criteria, the mean RBP test result for the triplicate tests was determined. If the mean result was not within the expected CV for the test (e.g., 100 ± 12% of the cumulative mean of the appropriate control value), the substance was deemed as “a potentially interfering substance” and would be tested in a dilution series until the concentration at which no interference had been determined. If a dilution series was performed, the dose-response relationship would be graphed. This method was based on Section 5.6 of NCCLS EP7-P.3

**Results**

In contrast to the reference mean values of 19.9 and 19.8 µg RBP/ml on assays 1 and 2, respectively, interfering substance values ranged from 18.1 to 20.7 µg RBP/ml and from 18.8 to 23.9 µg RBP/ml, respectively. This corresponded to 0.92 to 1.08 of the reference mean values, and was within expected CVs of the test.

These results are summarized in Table 5.3, in which values are expressed as the test (spiked) sample result divided by the reference (normal) sample result (T/R). SD and CV were calculated as averages of all RBP-EIA determinations for the interfering substances.

### Table 5.2. Spike Concentrations for RBP-EIA Interference Studies

<table>
<thead>
<tr>
<th>Spiked Substance</th>
<th>Test Concentration</th>
<th>Sigma Source Catalog No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein*</td>
<td>20% CRP</td>
<td>S2985</td>
</tr>
<tr>
<td></td>
<td>40% CRP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80% CRP</td>
<td></td>
</tr>
<tr>
<td>Rheumatoid factor†</td>
<td>20% RF</td>
<td>S3145</td>
</tr>
<tr>
<td></td>
<td>40% RF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80% RF</td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td>15 mg/dl</td>
<td>B4126</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>10 mg/ml</td>
<td>H7379</td>
</tr>
<tr>
<td>Human red blood cells</td>
<td>1%</td>
<td>R0043 and Fresh Finger Prick</td>
</tr>
<tr>
<td>Triglycerides as triolein</td>
<td>20 mg/ml</td>
<td>T7140</td>
</tr>
<tr>
<td>L-thyroxine</td>
<td>250 ng/ml</td>
<td>T2376</td>
</tr>
<tr>
<td>Vitamin A (retinol)</td>
<td>1 µg/ml</td>
<td>V7763</td>
</tr>
<tr>
<td>Estrone acetate</td>
<td>1 µg/ml</td>
<td>E7132</td>
</tr>
<tr>
<td>ȳ-Estradiol</td>
<td>1 µg/ml</td>
<td>E8875</td>
</tr>
<tr>
<td>Trans-thyretin</td>
<td>800 µg/ml</td>
<td>P7528</td>
</tr>
</tbody>
</table>

* Test concentrations represent percent increase over normal CRP values.
† Test concentrations represent percent increase over normal RF values.
Table 5.3. Interfering Substances Data

<table>
<thead>
<tr>
<th>Analyte and Concentration</th>
<th>Assay 1</th>
<th>Assay 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBP µg/ml</td>
<td>Spike Result T/R</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>21.8</td>
<td>1.04</td>
</tr>
<tr>
<td>Rheumatoid factor</td>
<td>20.8</td>
<td>1.00</td>
</tr>
<tr>
<td>Bilirubin, 15 mg/dl</td>
<td>20.5</td>
<td>1.03</td>
</tr>
<tr>
<td>Hemoglobin, 10 mg/ml</td>
<td>19.8</td>
<td>0.99</td>
</tr>
<tr>
<td>Human red blood cells, 1%</td>
<td>18.1</td>
<td>0.90</td>
</tr>
<tr>
<td>Triglycerides, 20 mg/ml</td>
<td>20.7</td>
<td>1.03</td>
</tr>
<tr>
<td>L-thyroxine, 250 ng/ml</td>
<td>19.1</td>
<td>0.96</td>
</tr>
<tr>
<td>Vitamin A, 1 µg/ml</td>
<td>20.4</td>
<td>1.02</td>
</tr>
<tr>
<td>Estrone acetate, 1 µg/ml</td>
<td>19.6</td>
<td>0.98</td>
</tr>
<tr>
<td>ß-estradiol, 1 µg/ml</td>
<td>20.5</td>
<td>1.03</td>
</tr>
<tr>
<td>Trans-thyretin, 800 µg/ml</td>
<td>20.4</td>
<td>1.08</td>
</tr>
<tr>
<td>Reference mean</td>
<td>19.9</td>
<td>1.00</td>
</tr>
<tr>
<td>SD</td>
<td>0.80</td>
<td>0.05</td>
</tr>
<tr>
<td>CV</td>
<td>4.2%</td>
<td>5.1%</td>
</tr>
</tbody>
</table>

Conclusions
None of the substances tested demonstrated a bias between the spiked and normal sera. All RBP values were within 12% and therefore are determined to be non-interfering. No dose-response titrations of the potential interfering substances were therefore necessary.

Field Evaluations

Studies to assess the RBP-EIA in comparison to the “gold standard” retinol-HPLC have been carried out using specimens acquired from VAD prevalence surveys in Nicaragua and in Cambodia.

Evaluation 1: Nicaraguan Sera

Methods
Ninety-two serum samples from mothers and children at risk for VAD were randomly selected from a larger group of specimens obtained from a population-based field study conducted by the Micronutrient Operational Strategies and Technologies (MOST) project in Managua, Nicaragua. Aliquots of sera were separated from whole blood specimens, which were then frozen and delivered to PATH. These samples were thawed and tested in PATH’s laboratory using:
1. PATH’s RBP-EIA kit;
2. RBP-RID test (The Binding Site, San Diego, CA); and
3. HPLC using a commercially available kit for retinol (HPLC Vitamin A/E, Bio-Rad, Hercules, CA).

Results
The data from these studies were compared in two-way analyses. The results are presented below as Figures 5.2 and 5.3. There was a very close correlation between the results of the RBP-EIA and retinol-HPLC ($R^2 = 0.82$) as well as an acceptable correlation between the results of the RBP-EIA and RBP-RID methods ($R^2 = 0.73$).

Figure 5.2. Evaluation of Retinol-HPLC Versus RBP-EIA on a Panel of Nicaraguan Sera (n=70)

Figure 5.3. Evaluation of RBP-EIA Versus RBP-RID on a Panel of Nicaraguan Sera (n=39)
Conclusions
The results from the field validation of retinol-HPLC and RBP-RID correlated closely with the results of the RBP-EIA. The retinol-HPLC to RBP-EIA correlation was stronger, as expected, since a commercially available kit offering a quantitative method was used. The RBP-RID method offered less precision, and reading the assay was considerably more subjective because precipitation rings formed by the RBP-anti-RBP complexes needed to be measured optically by a jeweler’s loupe over a back-lighted illumination box.

The researchers noted that the assays were much more closely correlated over the lower assay range where VAD would be expected and were weaker in the upper assay range where RBP levels are normal and indicate vitamin A sufficiency. In addition, they found that the commercially manufactured Bio-Rad kit for HPLC determination of retinol was accurate and very easy to use. It required a minimum of sample volume and was relatively simple and rapid in its operation. The kit, unfortunately, is not currently approved by the USFDA and therefore is not available for purchase in the United States.

Evaluation 2: Cambodian Sera

Methods
RBP-EIA and retinol-HPLC levels were determined for 359 individual serum specimens obtained in Cambodia during a national VAD survey conducted by Helen Keller Worldwide (HKW). Retinol was measured by HPLC using the commercial Bio-Rad Vitamin A/E kit. World Health Organization (WHO) criteria were used to classify VAD based on serum retinol, with the same cutoff extended to the classification of VAD using RBP.4

Results
Using WHO criteria to classify deficiency, 22.3% (95% CI, 18%, 26.6%) of this population was found to be moderately to severely deficient in serum retinol (Table 5.4).

<table>
<thead>
<tr>
<th>VAD level</th>
<th>(%)</th>
<th>95% CI</th>
<th>(%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe deficiency (&lt;0.35 µmol/L)</td>
<td>2.2</td>
<td>(0.7, 3.8)</td>
<td>0.6</td>
<td>(0.0, 1.3)</td>
</tr>
<tr>
<td>Moderate deficiency (0.35-0.70 µmol/L)</td>
<td>20.1</td>
<td>(15.9, 24.2)</td>
<td>20.3</td>
<td>(16.2, 24.5)</td>
</tr>
<tr>
<td>Vitamin A sufficient (&lt;0.70 µmol/L)</td>
<td>77.7</td>
<td>(73.4, 82.0)</td>
<td>79.1</td>
<td>(74.9, 83.3)</td>
</tr>
</tbody>
</table>
Using criteria previously established by Gamble et al. to classify deficiency,\(^5\) 20.9% (95% CI, 16.7% - 24.1%) of this population was found to have VAD based on serum RBP, while 22.3% were classified with VAD based on Retinol.

Defining VAD as \(\leq 0.70 \mu\text{mol/L}\) for both retinol and RBP, 80 subjects were identified with VAD using retinol as an indicator, while RBP-EIA identified 75 subjects. There was no significant difference in the proportion of the study population that was identified as deficient by either test.

**Accuracy of RBP-EIA as compared to serum retinol determined by HPLC**

Using a cutoff value of 0.70 \(\mu\text{mol/L}\) for both RBP and retinol to classify VAD, the sensitivity and specificity of the RBP-EIA were determined as 70% and 93.2%, respectively (Table 5.5). To illustrate the correlation, the RBP-EIA values were plotted against their corresponding retinol-HPLC values and are presented as Figure 5.4.

**Considering alternate cut-off values**

Additional performance characteristics were considered to further assess how the RBP-EIA performed compared to serum retinol by HPLC. A receiver operating characteristic (ROC) curve was produced (Figure 5.5) for RBP concentrations and serum retinol HPLC determinations at different cutoff points. An ROC curve plots the true positive rate (sensitivity) and false positive rate (1-specificity) of a test with continuous results and can be useful in helping to identify the cutoff that leads to an optimal combination of sensitivity and specificity for a test relative to a “gold standard.” (See Figure 5.5.)

### Table 5.5. Accuracy of RBP in Identifying Low Serum Retinol (n=359)

<table>
<thead>
<tr>
<th>Serum RBP</th>
<th>Serum Retinol (\leq 0.70 \mu\text{mol/L})</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>PPV (95% CI)</th>
<th>NPV (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>56</td>
<td>19</td>
<td>70.0% (58.7, 79.7%)</td>
<td>93.2% (89.7, 95.9%)</td>
<td>74.7%</td>
</tr>
<tr>
<td>-</td>
<td>24</td>
<td>260</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note:** PPV = Positive Predictive Value.

NPV = Negative Predictive Value.
A summary statistic that is also useful when comparing the RBP-EIA to the serum HPLC is the area under the ROC curve. An ROC curve of an ideal test would plot a true positive rate equal to 1.0 and a true negative rate equal to zero and would, therefore, have an area under the curve equal to 1.0. A relatively uninformative or inaccurate test would have an area under the curve of 0.5 or less. The ROC plot for the RBP-EIA compared with retinol-HPLC was calculated as 0.924.
Conclusion
This larger-scale field evaluation with 359 specimens from children in an at-risk population demonstrated a close relationship between serum retinol and RBP as quantified by retinol-HPLC and RBP-EIA. An equal proportion of subjects was found to have suboptimal vitamin A status when evaluated by EIA and HPLC (20.9% versus 22.3%, respectively). Sensitivity and specificity of the RBP-EIA were calculated at 70% and 93%, respectively. These values, we believe, reflect a normal and expected level of assay variation for both the reference HPLC and test EIA methods. The ROC curve discussed above may be more appropriate for this comparison. The calculated area under the curve, at 0.924, suggests that the RBP-EIA, when compared with retinol-HPLC, will be a useful test.

Conclusions
Following the guidelines in The United States Pharmacopoeia and The National Formulary, the RBP-EIA performance characteristics—including accuracy, intra-assay variability, precision, detection limit, quantitation limits, linearity, range, and analyte recovery—met or exceeded the product specifications. Interfering substances testing conducted under these guidelines showed no significant changes in the RBP-EIA’s performance when it was challenged with greater than normal levels of the substances.

In the study using a limited number of specimens from Nicaragua, the results of the RBP-EIA correlated closely with the results involving both the detection of retinol by HPLC and the detection of RBP by RID. The RBP-EIA and retinol-HPLC values correlated closely because a highly quantitative commercial kit was used. The RBP-RID method was considerably more difficult and subjective to interpret as well as more prone to procedural errors, but also correlated well. Both studies indicate that RBP could be used as a surrogate marker in specimens obtained from the field.

Data from the field evaluation performed on Cambodian sera indicate an excellent relationship between the serum retinol and RBP concentrations as quantified by HPLC retinol and RBP-EIA. These data further suggest that RBP as quantified by the RBP-EIA is an acceptable surrogate marker for retinol in estimating VAD in at-risk populations.
References


About This Section

PATH's Vitamin A team has developed the following troubleshooting guide to address problems that may arise in the laboratory. The RBP-EIA product insert (Appendix B) provides detailed information on obtaining appropriate specimens, performing the test, and determining the results.

## Initial Troubleshooting:
Confirm That Equipment Meets Proper Conditions and Procedures

<table>
<thead>
<tr>
<th>General Considerations</th>
<th>Pipetters</th>
<th>Manual microplate washer</th>
<th>Automated microplate washer</th>
<th>Microplate reader</th>
<th>Specimens</th>
<th>Specimen temperature</th>
<th>Reagent temperature</th>
<th>Specimen dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pipetters should be calibrated regularly and should be in good working condition. Disposable pipette tips must fit snugly.</td>
<td>Ensure that all dispenser nozzles are clear and that wash is delivered to wells equally.</td>
<td>Confirm that washer is programmed appropriately and functioning properly. Ensure that washer is dispensing and aspirating correctly. Adhere to manufacturer's maintenance recommendations.</td>
<td>Confirm that reader is functioning properly. Ensure that a 450-nm filter is used to read the assay plate. Adhere to manufacturer's maintenance and calibration recommendations.</td>
<td>Specimens must be serum. If not tested within 24 hours of collection, specimens must be frozen. Specimens may undergo up to 5 freeze/thaw cycles.</td>
<td>Allow specimens to equilibrate to room temperature (18° to 25°C) prior to testing. Thoroughly mix specimens before testing.</td>
<td>Allow reagents to equilibrate to room temperature (18° to 25°C) prior to testing. Thoroughly mix reagents before use.</td>
<td>Calibrators, controls, and patient specimens are diluted 1:25 in sample diluent. Thoroughly mix diluted specimens.</td>
</tr>
<tr>
<td>General Considerations</td>
<td>Conjugate reagent preparation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Accurately dilute conjugate concentrate 1:100 with sample diluent. Mix gently. Use clean, disposable containers for reagent preparation and delivery. Do not reuse containers. Add prepared conjugate reagent within 1 hour of preparation.</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Specimen/reagent delivery</td>
<td>Accurately deliver 100 µl of diluted specimens into bottom of microwells. Accurately dispense the appropriate volume of each reagent into top of microwells using a multi-channel pipetter.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubation temperature</td>
<td>Maintain recommended incubation temperature (18° to 25°C) throughout incubation.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubation timing</td>
<td>Adhere to incubation timing outlined in the package insert.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wash solution preparation</td>
<td>Accurately dilute wash concentrate 1:10 with deionized or distilled water. Mix the diluted wash solution for 5 minutes with a stir bar. Note: prepared wash solution is stable at room temperature for up to one month.</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plate washing</td>
<td>Wash assay plate 5 times with 350 µl of wash solution per well for each wash. Upon completion of the wash procedure, immediately add TMB substrate.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMB substrate</td>
<td>Use clean disposable containers for TMB delivery. Do not reuse containers. Add TMB substrate within 10 minutes of removal from the reagent bottle.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Data reduction</td>
<td>Use a linear curve fitting equation.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specimen results exceeding</td>
<td>Specimens exceeding 40 µg/ml RBP must be pre-diluted with sample diluent prior to diluting 1:25 and retested as described in the package insert.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Advanced Troubleshooting: Identify Symptom and Possible Cause

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Possible Cause</th>
<th>Correct Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration curve does not meet package insert specifications</td>
<td>Reagents used prior to reaching room temperature.</td>
<td>Warm reagents to room temperature (18° to 25°C) prior to starting assay procedure.</td>
</tr>
<tr>
<td></td>
<td>Assay plate allowed to dry during the plate-washing procedure.</td>
<td>Perform plate washes consecutively without allowing drying between washes or before adding TMB substrate.</td>
</tr>
<tr>
<td></td>
<td>Assay plate allowed to dry after completion of the wash procedure.</td>
<td>Upon completion of the wash procedure, immediately add TMB substrate to the assay plate.</td>
</tr>
<tr>
<td></td>
<td>Assay performed outside of recommended time/temperature ranges.</td>
<td>Assay procedure should be completed at 18° to 25°C. Refer to package insert for recommended timing of incubations.</td>
</tr>
<tr>
<td></td>
<td>Assay plate washed with improperly prepared wash solution or solutions other than diluted wash solution.</td>
<td>Wash solution should be prepared by diluting wash concentrate 1:10 with deionized water.</td>
</tr>
<tr>
<td></td>
<td>Containers used to prepare conjugate reagent reused.</td>
<td>Use only new, clean, disposable containers with tight-fitting lids. Discard after use.</td>
</tr>
<tr>
<td></td>
<td>Reagent boats used to add reagents to the assay plate with a multi-channel pipetter reused.</td>
<td>Use only new, clean, disposable reagent boats. Discard after use.</td>
</tr>
<tr>
<td></td>
<td>Assay plate read at incorrect wavelength.</td>
<td>Read plates at 450 nm.</td>
</tr>
<tr>
<td></td>
<td>Inadequate or incomplete plate washing.</td>
<td>Wash each well 5 times with a minimum of 350 µl of properly prepared wash solution dispensed per well for each wash cycle.</td>
</tr>
<tr>
<td></td>
<td>Excessive calibrator, control, and/or sample volume added to wells.</td>
<td>Add 100 µl of each diluted calibrator, control, and sample to the assay plate.</td>
</tr>
<tr>
<td></td>
<td>Use of unmatched 96-well assay plates and conjugate reagent.</td>
<td>Do not interchange reagents from different lots of RBP-EIA kits.</td>
</tr>
<tr>
<td></td>
<td>Incorrect dilution of conjugate concentrate into conjugate diluent.</td>
<td>Prepare conjugate reagent per package insert instructions.</td>
</tr>
</tbody>
</table>
### Advanced Troubleshooting: Identify Symptom and Possible Cause

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Possible Cause</th>
<th>Correct Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration curve does not meet package insert specifications (continued)</td>
<td>Preparation of conjugate reagent more than 1 hour prior to use.</td>
<td>Prepare conjugate reagent prior to pipetting calibrators, controls, and samples. Add to assay plate within 1 hour of preparation.</td>
</tr>
<tr>
<td></td>
<td>Insufficient volume of conjugate reagent added to assay plate.</td>
<td>Add 100 µl of conjugate reagent to each well of the assay plate.</td>
</tr>
<tr>
<td></td>
<td>Pouring TMB substrate solution into reagent boat more than 10 minutes prior to use.</td>
<td>Pour TMB substrate solution during last 5 minutes of conjugate reagent incubation. Add to assay plate within 10 minutes of removal from the reagent bottle.</td>
</tr>
<tr>
<td></td>
<td>Insufficient volume of TMB substrate solution added to assay plate.</td>
<td>Add 200 µl of TMB substrate solution to the assay plate.</td>
</tr>
<tr>
<td></td>
<td>Assay performed outside of recommended time/temperature ranges.</td>
<td>Assay procedure should be performed at 18° to 25°C. Refer to package insert for recommended timing of incubations.</td>
</tr>
<tr>
<td></td>
<td>Assay plate incubated on an automated shaker.</td>
<td>Assay plate should be incubated without shaking.</td>
</tr>
<tr>
<td>Calibrator absorbency values higher than typical or greater than 3.0 OD units</td>
<td>Incorrect dilution of conjugate concentrate into conjugate diluent.</td>
<td>Prepare conjugate reagent per package insert instructions.</td>
</tr>
<tr>
<td></td>
<td>Excess conjugate reagent added to assay plate.</td>
<td>Add 100 µl of conjugate reagent per well of the assay plate.</td>
</tr>
<tr>
<td></td>
<td>Inadequate or incomplete plate washing.</td>
<td>Wash each well 5 times with a minimum of 350 µl of properly prepared wash solution dispensed per well for each wash cycle.</td>
</tr>
<tr>
<td></td>
<td>Excess TMB substrate added to assay plate.</td>
<td>Add 200 µl of TMB substrate to the assay plate.</td>
</tr>
<tr>
<td></td>
<td>Assay plate allowed to dry during the plate washing procedure.</td>
<td>Do not allow plates to dry before addition of TMB substrate.</td>
</tr>
<tr>
<td></td>
<td>Assay plate allowed to dry after completion of the wash procedure.</td>
<td>Upon completion of the wash procedure, immediately add TMB substrate to the assay plate.</td>
</tr>
<tr>
<td></td>
<td>Use of a single-channel or Eppendorf repeater pipettet for reagent addition.</td>
<td>Dispense all reagents with an 8-channel pipettet.</td>
</tr>
</tbody>
</table>
## Advanced Troubleshooting: Identify Symptom and Possible Cause

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Possible Cause</th>
<th>Correct Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor precision between sample duplicates</td>
<td>Inadequate or incomplete plate washing.</td>
<td>Wash each well 5 times with a minimum of 350 µl of properly prepared wash solution dispensed per well for each wash cycle.</td>
</tr>
<tr>
<td></td>
<td>Poor pipetting technique with 8-channel pipetter during stopping reagent addition, causing formation of green color in wells.</td>
<td>Dispense stopping reagent with 8-channel pipetter using a decisive motion to allow adequate mixing of reagents in each well.</td>
</tr>
<tr>
<td></td>
<td>Green color in the wells after addition of stopping reagent, even though correct pipetting technique followed.</td>
<td>The stopping solution has become unstable or diluted. Prepare fresh 1% weight-to-volume HCl and repeat assay.</td>
</tr>
<tr>
<td></td>
<td>Use of a permanent tip pipetter for calibrator, control, and sample addition.</td>
<td>Use a disposable tip pipetter.</td>
</tr>
<tr>
<td></td>
<td>Calibration curve does not meet package insert specifications.</td>
<td>Refer to package insert for assay validation specifications.</td>
</tr>
<tr>
<td></td>
<td>Incorrect data-reduction method used to calculate assay results (e.g., point-to-point).</td>
<td>Use a linear curve-fitting equation. Refer to package insert for duplicate precision specifications.</td>
</tr>
<tr>
<td></td>
<td>Poor precision between sample duplicates.</td>
<td>Refer to package insert for duplicate precision specifications.</td>
</tr>
<tr>
<td>Control values outside specified ranges</td>
<td>Incorrect calibrator dilution.</td>
<td>Follow package insert directions for preparation of calibrators from the material supplied with kit.</td>
</tr>
<tr>
<td></td>
<td>Incorrect control volume added to assay plate.</td>
<td>Add 100 µl of each diluted control to the assay plate.</td>
</tr>
<tr>
<td></td>
<td>Assay performed at temperatures above recommended room temperature range.</td>
<td>Perform assay procedure at 18° to 25°C.</td>
</tr>
<tr>
<td></td>
<td>Wash solution prepared with stopping reagent instead of 10x wash concentrate.</td>
<td>Prepare fresh wash solution per package insert instructions. Thoroughly flush washer with deionized water and prime fresh wash solution.</td>
</tr>
<tr>
<td></td>
<td>Conjugate concentrate was not added to sample diluent.</td>
<td>Prepare conjugate reagent solution per package insert instructions.</td>
</tr>
<tr>
<td>No color development after TMB substrate</td>
<td>Solution other than TMB substrate was added to the plate.</td>
<td>Follow package insert instructions for reagent addition to plate.</td>
</tr>
</tbody>
</table>
Section 7

Performance Highlights of the RBP-EIA

Simple
In developing countries, semiskilled laboratory personnel can perform the assay in laboratories near the study site.

Rapid
Once the specimens have been transferred to the strip wells, the assay takes 35 to 40 minutes to complete one plate containing 96 samples (48 samples if assayed in duplicate).

Accurate and Repeatable
Accuracy: 0.96 ± 0.04%. Intra-assay variability: 8.9% CV. This test will accurately forecast the vitamin A status in a population.

Precise
Precision: 6.25% CV in the calibrated range. This test will quantify serum RBP levels in a sample within 6.25% in the assay’s calibrated range.

Quantitative
The test is accurate within a calibrated range of 10 to 40 µg/ml of RBP. This range represents vitamin A status from severely deficient through normal vitamin A levels.

- **Detection limit:** The assay can distinguish RBP levels to a minimum of 1.1 µg/ml intervals in sample RBP concentrations.

- **Quantitation limit:** The quantitation limit is the lowest amount of RBP that can be detected with accuracy and precision. The quantitation limit of the RBP-EIA is 7.7 µg/ml of RBP.

- **Range:** The range of an assay is the area between lowest concentration and the highest concentration of RBP that provides accurate results. The range of the RBP-EIA is 10 to 40 µg/ml RBP.

Sensitive and Specific
The test is able to identify the cutoff points that are indicative of moderate to severe VAD, and identifies the truly deficient percentage of the population. The test detects only RBP. An interference testing study resulted in no RBP values with the substances evaluated.
Next Steps

As the previous sections have shown, PATH’s RBP-EIA represents an important development for efforts to assess clinical VAD on population levels. Activities to refine and commercialize the test are currently underway and will maximize the RBP-EIA’s impact on programs working to reduce the prevalence of VAD.

Future Research and Test Development

PATH plans to conduct research on the RBP-EIA and refinements of the test. Next steps include:

- **Investigating the use of DBS as a potential sample for the RBP-EIA.** Studies are in progress to investigate:
  - optimal drying and storage conditions for DBS;
  - differences between DBS made from venous or capillary blood;
  - effect of hematocrit on the assay and RBP concentrations;
  - serum stability.

- **Conducting field validation of the data.** If the DBS results are promising, larger-scale field evaluations will be conducted in one or more developing country settings using DBS as the mode of specimen collection.

- **Conducting retrospective and prospective studies on serum RBP levels.** PATH is conducting a retrospective study in collaboration with Dr. Antonio Quiros from Children’s Hospital, Los Angeles. The study has produced preliminary data that suggest that serum RBP levels can be used to monitor vitamin A uptake and onset of liver failure in patients on long-term perinatal nutrition (PN). Dr. Quiros will continue to investigate serum RBP levels in a prospective study with patients on PN for at least three months, compared to the routine PN liver-disease assessment monitoring assays. The purpose of this study is to determine if serum RBP levels correlate with liver biosynthetic activity regardless of serum vitamin A levels during PN, and if serum RBP levels will continue to predict which patients are at risk of developing PN-related liver disease. After recruiting eligible patients, Dr. Quiros will complete the work as described in his submitted abstract, “Serum Retinol Binding Protein Determinations as a Diagnostic Tool in Perinatal Nutrition Associated Liver Disease,” which was presented at the 2001 Interdisciplinary Neonatal Nutrition Symposium. Dr. Quiros has also proposed a study to monitor host acceptance or rejection post-liver-transplantation using the RBP-EIA.
Commercial Availability

PATH has chosen Scimedx Corporation as its commercial partner for the RBP-EIA. While the test is not currently available for purchase, Scimedx is confident that the test will be commercially available by fall 2003.
Appendix A
1. RBP-ELISA (Immundiagnostik GmbH)
2. RBP-RID (Binding Site)
3. Retinol-HPLC (Bio Rad)

Appendix B
RBP-EIA Product Insert

Appendix C
Use of Dried Blood Spots as Specimens for the RBP-EIA
Retinol Binding Protein

ELISA

For the in vitro determination of Retinol binding protein (RBP) in plasma, serum and urine

(Only for research purpose)

Art. Nr: K 6110
Packagesize: 96 tests
Storage: 2-8 °C

Tel.: 06251-39082
Fax: 06251-39084

Immundiagnostik GmbH · Wiesenstraße 4 · D-64625 Bensheim
**Test principle:**

This Enzyme-Linked Immunosorbent Assay (ELISA) serves the quantitative determination of the Retinol binding protein (RBP) from plasma, serum and urine. In a 1 hour incubation step, the RBP in the samples is bound to an available excess of polyclonal rabbit antibodies against RBP, which is immobilized to the surface of the microtitre plates. After a washing step, to remove all foreign substances, the quantification of bound RBP is carried out by adding an enzyme labeled antibody, which also binds to the RBP. The amount of converted substrate is directly proportional to the amount of bound RBP and can be determined photometrically at 450 nm (if extinction is out of range measure at 410 nm).

**Reagents in the test package:**

- 1 microtitre plate
- 20 μl 1. antibody (rabbit-anti RBP)
- 6 x standard solutions (670, 220, 74, 25, 8, 0 μg/l), ready to use
- 20 μl PO-antibody (Peroxidase-labelled)
- 11 ml TMB substrate solution **Caution: avoid contact with skin**
- 10 ml stop solution
- 10 ml coating buffer
- 25 ml blocking reagent
- 2 x 50 ml dilution buffer
- 10 ml NaCl (0.9 %)
- 50 ml washing buffer concentrate
- 40 μl control, ready to use
Sample preparation

Plasma or serum: Samples can be stored for two weeks at 4°C. They should be frozen when stored longer. **The samples should be diluted 1:500 in dilution buffer before use** (20 µl sample in 1 ml dilution buffer, then dilute 50 µl of this solution with 450 µl dilution buffer).

Urine: Adjust the urine to a pH of 6 to 8 with 1 N NaOH and store samples at -20°C until testing. Dilute samples with an RBP content of more than 670 µg/L 1:10 with dilution buffer.

Required laboratory equipment

- Closing film for microtitre plates
- Photometer with filter 450 nm
- Horizontal mixer
- Pipettes: 10µl, 100µl, 200µl, 500µl and multipette
Preparation of reagents

⇒ Dilute the washing buffer 1:10 in destilled or deionized water (50 ml concentrate + 450 ml destilled water).

⇒ Dilute the 1. antibody (rabbit-anti RBP) 1:1000 in coating buffer (10 µl 1. antibody in 10 ml buffer).

⇒ The standards and the control should be stored for 14 days at 2-8°C. After this time they should be stored at -20 °C.

⇒ Dilute the PO-antibody 1:1000 in washing buffer (10 µl PO-antibody in 10 ml washing buffer).

Normal range

<table>
<thead>
<tr>
<th></th>
<th>Plasma or serum:</th>
<th>Urine:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 - 75 mg/L</td>
<td>0,01 - 0,54 mg/L</td>
</tr>
</tbody>
</table>
Assay procedure

Carry out the tests in duplicate in the supplied microtitre plate.

✓ Pipette 100 μl of diluted 1. Antibody in each cavity of the microtitre plate and incubate for 1 hour at room temperature, shaking on a horizontal mixer.

✓ Decant the content of the plate and wash the cavities 3 x with 200 μL of washing buffer.

✓ Pipette 200 μl blocking reagent in each cavity and incubate for 30 minutes at room temperature, shaking on a horizontal mixer.

✓ Decant the content of the plate and wash the cavities 3 x with 200 μl of washing buffer.

✓ Pipette 100 μl of 0.9% NaCl solution into each cavity of the microtitre plate.

✓ Add 10 μl of standard solutions (0, 8, 25, 74, 220 and 670 μg/l), patient samples plasma and serum (preparation s.S.2) or urine.

✓ Incubate for at least 1 hour at room temperature, shaking on a horizontal mixer.

✓ Decant the content of the plate and wash the cavities 5 x with 200 μl of washing buffer.

✓ Add 100 μl diluted PO-antibody in each cavity.

✓ Incubate for at least 1 hour at room temperature, shaking on a horizontal mixer.

✓ Decant the content of the plate and wash the cavities 5 x with 200 μl of washing buffer.

✓ Add 100 μl of TMB substrate solution

✓ Incubate for 10-20 minutes at room temperature, shaking slightly, until color differences are sufficient.

- Add 50 μl of stop solution and mix shortly.

- Measure the extinction of the samples at 450 nm directly after adding the stop solution and mixing.
Quality control:

Typical standard curve:

Detection limit:

The detection limit of this RBP ELISA was determined to $B_0 + 2SD$. The limit is 0.01 mg/l.

Linearity:

The linearity of this assay was detected through dilutions with dilution buffer of material which contains RBP. The linearity is extended from 0.01 - 0.67 mg/l.
Within and between assay:

Through repeated measurements (n=16) of material which contains RPB the following results were obtained:

<table>
<thead>
<tr>
<th>mean [mg/l]</th>
<th>CV within [%]</th>
<th>CV between [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>6.8</td>
<td>7.9</td>
</tr>
</tbody>
</table>
General notes on the test and test procedure

- The test components which are made of human serum are tested for Australia antigen and HIV and found to be negative. However, since no test method can offer complete assurance that infectious agents are absent, these reagents should be handled as recommended for any potentially infectious human serum or blood specimen. The normal precautions for laboratory working should be observed.

- Reagents of the test package contain sodium azide as a bactericide. Contact with skin or mucous membranes is to be avoided.

- All reagents in the test package are to be used only for in-vitro diagnostics.

- The reagents should not be used after the date of expiry (see label on the test package).

- Single components with different lot numbers should not be mixed or exchanged.

- For quality control, the guidelines for medical laboratories should be observed.

- The characteristic test data, such as incubation time, incubation temperature and pipetting volumina of the different components are defined by the producer. Any variations of the test procedure, that are not coordinated with the producer, may influence the results of the test. Our company can therefore not be held reliable for any damage resulting from this.

Effective 25.03.97 Revised
HUMAN RETINOL BINDING PROTEIN 'NL'

NANORID™ RADIAL IMMUNODIFFUSION KIT

For In Vitro Diagnostic Use Only

Product Code: GD117.3  Document Code: RIM186.5 pl of 14

1. INTENDED USE

This kit is for quantitating retinol binding protein in human serum as an aid in diagnosing kidney disease.

2. SUMMARY AND EXPLANATION:

Retinol binding protein (RBP) is a low molecular weight (21kDa) protein that is involved in the binding of retinol (vitamin A alcohol) and its transport from the liver. It exists in serum mainly as a complex with transthyretin (prealbumin), which prevents its glomerular filtration and renal catabolism.

Increased serum levels of RBP are associated with renal failure, due to reduced filtration. Increased urine concentrations can also occur due to impaired tubular uptake. Reduced serum RBP concentrations are associated with acute malnutrition, vitamin A deficiency, liver disease and a number of other conditions (refs 1-3).

Radial immunodiffusion (RID) is a technique that is routinely used for measuring the concentration of various soluble antigens in biological fluids. It is principally derived from the work of FabeY & McKelvey (ref. 4) and Nancini et al. (refs. 5 & 6).

3. PRINCIPLE:

The method involves antigen diffusing radially from a cylindrical well through an agarose gel containing an appropriate mono-specific antibody. Antigen-antibody complexes are formed which, under the right conditions, will form a precipitin ring. The ring size will increase until equilibrium is reached between the formation and breakdown of these complexes, this point being termed 'completion'. At this stage, a linear relationship exists between the square of the ring diameter and the antigen concentration. By measuring the ring diameters produced by a number of samples of known concentration, a calibration curve may be constructed. The concentration of the antigen in an unknown sample may then be determined by measuring the ring diameter produced by that sample and reading off the calibration curve.

(NANORID™ kits are patented products).

CDC/FDA (USA) Information: Complexity Cat: High
Analyte ID Code: 5507
Test System ID Code: 61253

Issue Date: April 1998
There are three different procedures that may be used with these kits (see Section 4D). Procedures ONE and TWO require that the rings are measured at completion. A linear calibration curve is constructed for Procedure TWO, whereas for Procedure ONE a reference table (based upon the ideal linear calibration curve) is provided, which converts ring diameters directly to protein concentrations. Using Procedure THREE, ring diameters are measured before completion; the calibration curve produced will be non-linear.

4. **REAGENTS:**

A. **RBP plates** (supplied in foil pouches). These contain monospecific antibody to RBP in agarose gel. Up to fourteen samples can be run per plate (including calibrator(s)). Preservatives: 0.1% sodium azide, 0.1% N-amo-n-caprylic acid (BACA), 0.01% thiomersal (sodium ethylmercurithiosalicylate), 0.01% benzamidine.

B. **Calibrator,** supplied lyophilised. The concentration of RBP is given on the vial label. Preservatives: 0.1% sodium azide, 0.1% BACA, 0.01% benzamidine.

C. **Control,** supplied lyophilised. The expected RBP concentration is given on the vial label. Preservatives: 0.1% sodium azide, 0.1% BACA, 0.01% benzamidine.

D. **7% bovine serum albumin (BSA) solution.** This is supplied in stabilised liquid form and is included for use as a diluent. Preservative: 0.1% sodium azide.

E. **Distilled Water** For reconstituting the lyophilised calibrator and control. Preservative: 0.1% sodium azide.

5. **CAUTION:**

All donors of human serum supplied in this kit have been tested and found negative for hepatitis B surface antigen (HBsAg) and antibodies to HIV and hepatitis C virus. However, these tests cannot guarantee the absence of infective agents. Proper handling and disposal methods should be established as for all potentially infective material and only personnel adequately trained in such methods should be permitted to perform the procedures.

The plates and other kit components contain 0.1% sodium azide and 0.01% thiomersal as preservatives. The usual precautions in handling and disposal should therefore be observed.

6. **STORAGE AND STABILITY:**

The unopened kits should be stored at 2-8°C and can be used until the expiry date given on the kit box label. DO NOT FREEZE. The expiry dates of individual components are given on the component labels. RBP plates should be stored at 2-8°C and are damaged by temperature extremes. Freezing will destroy the gel, therefore RBP plates should be kept away from cooling elements in refrigerators. High temperatures should also be avoided as this will result in moisture loss from the gel, affecting performance. Unopened plates should be stored flat and upside down (pouch label uppermost) to prevent condensation accumulating in the wells. Handle plates with care to prevent gel damage.

The lyophilised calibrator and control should be stored at 2-8°C. Once reconstituted they are stable for at least one week at 2-8°C, but for longer storage they should be aliquotted and frozen. (Do not store in a self-defrosting freezer). All other reagents should be stored at 2-8°C.
7. SPECIMEN COLLECTION AND PREPARATION:

Serum samples should be used for this assay. They should either be fresh (store at 2-8°C for up to 8 hours) or deep frozen (-20°C or below). The RBA included in the kit should be used as diluent when required, as this will maintain the viscosity of the material. Results can therefore be accurately compared with the calibrator which has a similar viscosity to normal Serum.

8. METHODOLOGY

(A summary of the entire procedure is given at the end of this instruction leaflet).

A Materials provided:

Code GD17.3
1. 3 x radial immunodiffusion plates in foil pouches
2. 2 x gel sectioning blades per plate
3. 1 x lyophilised calibrator
4. 1 x lyophilised control
5. 4 x 3ML 75% BSA
6. 2 x 3ML distilled water
7. 1 x instruction leaflet, including RID reference table/graph paper.

B Materials required but not provided:

1. Equipment for collection and preparation of test samples, eg sample tubes, centrifuge etc.

2. Pipettes for accurate dilution of samples and reconstitution of calibrator(s) and control(s).

3. Micropipettes for sample application. These should be capable of accurately delivering 5uL volumes. Siringe Micropipettes (Code AD041) or "Hamilton" syringes are recommended.

4. Jewellers' Eyepiece (Code AD040) or Electronic RID Reader (Codes AD001 or AD030) for magnifying and accurately measuring the precipitin ring diameters to 0.1mm.

C Reagent Preparation

1. RID Plate(s)

To avoid contamination of the gel, plates should be used in a dust-free environment. Take the plate from the foil pouch and remove the lid. If condensation is visible the plate should be kept upside down until the lid has been removed to prevent droplets falling onto the gel. Check the plate to ensure that no damage has occurred in storage or transit, eg splits in the gel. Leave the plate open for 10-15 minutes (or longer if necessary) at room temperature to allow any condensation in the well or on the gel surface to evaporate. Samples should never be applied to wells in which moisture is still visible.

Plate partitioning: The plates may be partitioned into up to four sections using the gel dividers provided. Each divider should be positioned carefully on the gel, cutting edge downward, with the stabilising arm resting on the central plate label. Press firmly on the arm to cut the gel and leave in position.
Plate partitioning is recommended if only part of the plate is to be used initially or when using suspected high concentration samples which could (by diffusing over a wide area) result in antibody depletion occurring elsewhere on the plate. After initial use, partitioned plates should be ressealed in their foil pouches and stored at 2-4°C with the gel divider(s) in place. Store partitioned plates right side up and use within four weeks.

(2) Calibrator(s)

The lyophilised calibrator should be reconstituted with the volume of distilled water indicated on the vial label - use the distilled water provided in the kit. Before use, all material in the bottle, including any adhering to the bung must be completely dissolved (by inversion) over a minimum period of thirty minutes. The calibrator is prediluted and should be applied to the plates neat. Dilutions of the calibrator must be made if a calibration curve is required (as for Procedures TWO and THREE). These dilutions should normally be a medium dilution (60%, i.e. 6 parts in 10) and a low dilution (10%, i.e. 1 part in 10). It is recommended that 120 μL of calibrator is mixed with 80 μL of the diluent provided (7% BSA) for a 60% dilution, and 25 μL of calibrator is mixed with 225 μL of the diluent for a 10% dilution.

(3) Control

The lyophilised control should be reconstituted with the volume of distilled water indicated on the vial label. This should be mixed gently by inversion until the contents are completely dissolved. It should be diluted 1/20 (1 part in 20). It is recommended that 10 μL of test sample is mixed with 190 μL of diluent (7% BSA). Mix gently before use.

(4) Samples

Samples should be diluted 1/20 (1 part in 20) before applying to the plates. To obtain adequate accuracy, it is recommended that 10 μL of test sample is mixed with 190 μL of diluent (7% BSA). Mix gently before use. If samples containing high protein concentrations are to be measured, a higher dilution factor will be necessary. In such cases it is suggested that to obtain adequate accuracy a minimum volume of 10 μL of test sample is mixed with the appropriate volume of BSA. For samples having very high protein concentrations below the detection limits of the plates, apply the samples undiluted.

D Procedures

(1) Procedure ONE: - RID Reference Table.

This method does not require the construction of a calibration curve - sample concentrations corresponding to each ring diameter are read directly off the RID Reference Table. Rings must be allowed to develop to completion which will require a minimum diffusion time of 96 hours. The neat calibrator should be run on each plate used to ensure all are performing correctly.

(2) Procedure TWO: - Calibration Curve at Completion

In this method, the neat calibrator plus the two dilutions are used to produce a linear calibration curve. Rings must be allowed to develop to completion which will require a minimum diffusion time of 96 hours. To conserve wells, one calibration curve can be used for several plates of the same batch used concurrently. In such cases, the neat calibrator should be run on each plate used to ensure all are performing correctly.
(3) Procedure THREE: Calibration Curve prior to Completion:

In this method, the next calibrator plus the two dilutions are used to produce a calibration curve which is non-linear, as the rings are measured before completion. The minimum recommended diffusion time is 18 hours. It is advisable that a separate calibration curve is constructed for each plate used.

E. Application of Calibrators and Samples

The calibrator (including the two dilutions if required), control and test samples (appropriately diluted) should be gently mixed immediately before use. Fill the required number of wells with 5 μL of the neat calibrator using a micropipette. If Procedure TWO or THREE is being followed, also fill the required number of wells with the medium and low calibrator dilutions. The remaining wells should then be filled with 5 μL of appropriately diluted test samples and the control. Plates should not be left open for long periods during calibrator/test sample application, as this will cause excessive drying of the gel.

F. Incubation

After sample application, the lid is tightly closed and the plate stored flat at room temperature (approximately 20-24°C). It is essential that the gel is not allowed to dry out during incubation. To minimise evaporation, it is suggested that plates should either be resealed in their foil pouches or stored in a moist box (a sealed plastic box containing damp tissue paper) during incubation. The minimum incubation time for Procedure THREE is 18 hours and for complete diffusion (Procedures ONE and TWO) is 36 hours. Final ring diameters may be affected by temperature; the expected ring size for the neat calibrator is 9mm (± 0.3mm) when incubated at 20-24°C. Extremes of temperature should be avoided.

G. Quality Control

The control should, following reconstitution, be treated exactly like a test sample. Values obtained for the control should be within 1% of the concentration stated on the vial label. For procedure ONE, the confidence limit is ± 0.3mm eg. If the RBP concentration quoted on the control vial is 5μg/mL, this is equivalent to a ring diameter of 6.8mm (from the RID reference table). The control should therefore give a ring diameter in the range 6.5-7.1mm.

9. RING MEASUREMENT AND RESULTS PROCESSING

After the required diffusion time, ring diameters should be measured to the nearest 0.1mm using a jeweller's eyepiece or a RID plate reader. When reading with an eyepiece, use bright side lighting and a dark background. If difficulties are experienced, view the plate macroscopically and mark the edges of the rings on the back of the plate using a needle. The distance between these marks may then be more easily measured.

Note: For Procedures ONE and TWO ring diameters must have developed to completion. If there is any doubt, rings should be remeasured after a further 24 hours to ensure there has been no increase in their diameters. The neat calibrator should give a ring diameter of 9.0mm ± 0.3mm at completion. If the ring diameter is outside this range, see Trouble Shooting (Section 10C).

Procedure ONE

The concentration of the RBP in each test sample can be read directly from the RID Reference Table, providing it has been applied diluted 1/20 as recommended.
Concentrations obtained for samples giving ring diameters greater than the neat calibrator should be regarded as approximate, due to the possibility of incomplete diffusion. Such samples may also cause local antibody depletion thereby affecting adjacent ring sizes; they should preferably be further diluted and retested. Samples giving ring diameters below the lower limit on the RID Reference Table should be retested in a less diluted form (see Section 8C (4)). Any change from the recommended sample dilution (i.e. 1/20) must be taken into account when calculating the results.

Example

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>Dilution</th>
<th>Ring Diameter (mm)</th>
<th>Table Value (mg/L)</th>
<th>Original Sample Conc. (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBP Serum</td>
<td>1/20</td>
<td>6.8</td>
<td>51.0</td>
<td>51.0</td>
</tr>
<tr>
<td>RBP Serum</td>
<td>1/20</td>
<td>&gt;11</td>
<td>&gt;156</td>
<td>&gt;156</td>
</tr>
<tr>
<td>RBP Serum</td>
<td>1/40</td>
<td>8.6</td>
<td>90.1</td>
<td>180.2*</td>
</tr>
</tbody>
</table>

* Calculated as follows: Table value x Recommended Diln./Actual Diln. i.e. 90.1 mg/L x (1/20)/(1/40).

Procedure TWO.

Plot the square of the diameters of the precipitin rings formed by the neat calibrator plus the two dilutions versus their concentrations (given on the neat calibrator vial label). RBP concentrations should be along the horizontal (x) axis, ring diameters squared along the vertical (y) axis. A line of best fit is drawn through the three points; the y-intercept should be in the range 10-12mm². The RBP concentration is determined from the calibration curve; remember to adjust the sample concentration obtained by the dilution factor used.
Sample Calculation:

RBP calibrators (i.e. the neat calibrator plus the two dilutions) gave the following ring diameters on a RBP test plate at completion:

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>Conc. (mg/L)</th>
<th>Diameter (D) of ring (mm)</th>
<th>D squared (mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neat</td>
<td>5.0</td>
<td>9.0</td>
<td>81.0</td>
</tr>
<tr>
<td>60%</td>
<td>3.0</td>
<td>7.2</td>
<td>51.8</td>
</tr>
<tr>
<td>10%</td>
<td>0.5</td>
<td>4.1</td>
<td>16.8</td>
</tr>
</tbody>
</table>

A calibration curve was plotted using these results:

An unknown sample, applied diluted 1/20 as recommended, gave a 6.6mm diameter ring on this plate. From the above curve, this corresponds to a RBP concentration of 2.43mg/L. Therefore, the RBP concentration in the undiluted sample = 2.43 x 20 = 48.6mg/L.

Procedure THREE

Plot the calibration curve as for Procedure TWO. The graph will not be a straight line but a curve, the gradient of which decreases with increasing protein concentration. The y-intercept should be as indicated for Procedure TWO. Test sample protein concentrations are read off the calibration curve; remember to adjust the sample concentration obtained by any dilution factor used.

Sample Calculation:

RBP calibrators (i.e. the neat calibrator plus the two dilutions) gave the following ring diameters on a RBP plate after 18 hours:

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>Conc. (mg/L)</th>
<th>Diameter (D) of ring (mm)</th>
<th>D squared (mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neat</td>
<td>5.0</td>
<td>7.9</td>
<td>62.4</td>
</tr>
<tr>
<td>60%</td>
<td>3.0</td>
<td>6.7</td>
<td>44.9</td>
</tr>
<tr>
<td>10%</td>
<td>0.5</td>
<td>4.1</td>
<td>16.8</td>
</tr>
</tbody>
</table>
An unknown sample, applied diluted 1/20 as recommended, gave a 6.1 mm ring on this plate. From the above curve, this corresponds to a RBP concentration of 2.32 mg/L. Therefore, the RBP concentration in the undiluted sample = 2.32 x 20 = 46.4 mg/L.

10. LIMITATIONS OF PROCEDURES

A. For Procedure ONX, results generated from ring diameters greater than the next calibrator ring diameter (i.e. 9 mm) should be regarded as approximate (See Section 9). For procedures TWO and THREE, accurate results are limited to the calibration curve between the next and low calibrator dilution values – extrapolation beyond these points is not valid. Samples giving results outside these ranges must be diluted or concentrated as appropriate and retested (See Section 8C (4)).

B. For CDC/FDA information - see front page of insert

C. Trouble Shooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Causes(s)</th>
<th>Suggested Action(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. No ring for:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Calibrator(s)</td>
<td>Calibrator omitted.</td>
<td>Repeat assay.</td>
</tr>
<tr>
<td>3. Calibrator(s) and test samples.</td>
<td>Plate deterioration.</td>
<td>a) Storage damage. Repeat assay using new plate. b) Product expired. Repeat assay using new plate/kit.</td>
</tr>
</tbody>
</table>

B. Oversize Rings for:

1. Heat calibrator (more than 9.3 mm) | (i) Inaccurate ring measurement. | Remeasure using eyepiece or RID Plate Reader. |
| | (ii) Incorrect volume applied. | Check 5uL volume applied. |
| | (iii) Inaccurate volume applied. | (a) Micropipette malfunction - check operation and repeat assay. |
| | | (b) Poor technique - repeat assay. |
(iv) Inaccurate calibrator reconstitution.

(a) Pipette malfunction - check operation and calibration, then repeat assay using new calibrator.
(b) Poor technique - repeat assay using new calibrator.

Partial evaporation of reconstituted calibrator on storage.

Repeat assay using new calibrator/kit.

(vi) Plate deterioration.

(a) Storage damage. Repeat assay using new plate.
(b) Product expired. Repeat assay using new kit.

dilute the sample(s) responsible and repeat assay using new plate.

Repeat assay, incubating at 22°C.

(vii) Local antibody depletion due to adjacent high concentration test samples.

Dilute and re assay. Check 5 μL volume applied.

(viii) Incubation temperature too high (see Section 8F).

2. Test samples (above acceptable range - see Section 10)

Concentration too high.

(i) Concentration too high.
(ii) Incorrect volumes applied.

Dilute and re assay. Check 5 μL volume applied.

C. Underestimated rings for:

1. Heat calibrator (less than 8.7 mm)

(i) Inaccurate ring measurement.
(ii) Incorrect volume applied.
(iii) Inaccurate volume applied.
(iv) Inaccurate calibrator reconstitution
(v) Calibrator deterioration.

(a) Storage damage. Repeat assay using new calibrator.
(b) Product expired. Repeat assay using new kit.

Repeat assay, incubating at 22°C.

(vi) Incubation temperature too low (see Section 8F).

2. Test samples (below acceptable range - see Section 10A)

Concentration too low.

(i) Concentration too low.
(ii) Incorrect volume applied.

See Section 8C(4) and repeat assay. Check 5 μL applied.

D. Double/Multiple rings

(i) Non-specific precipitation close to well (due to PBS in well).
(ii) Poor sample application.
(iii) Calibrator deterioration

Read outer ring.

(a) Storage damage. Repeat assay using new calibrator.
(b) Product expired. Repeat assay using new kit.
<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause(s)</th>
<th>Suggested Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. Non-circular rings</td>
<td>(i) Poor sample application.</td>
<td>Repeat assay.</td>
</tr>
<tr>
<td></td>
<td>(ii) Gel dried out before use.</td>
<td>Repeat assay using fresh sample.</td>
</tr>
<tr>
<td></td>
<td>(iii) Gel dried out during sample application or incubation.</td>
<td>Repeat assay.</td>
</tr>
<tr>
<td></td>
<td>(iv) Local antibody depletion (due to high concentration samples on the plate.)</td>
<td>Repeat assay minimising the time the plate is left open.</td>
</tr>
<tr>
<td>F. Cloudy gel</td>
<td>(i) Plate has been frozen.</td>
<td>Repeat assay using new plates. Review storage.</td>
</tr>
<tr>
<td></td>
<td>(ii) Gel dried before use.</td>
<td>As for E (ii) above.</td>
</tr>
<tr>
<td></td>
<td>(iii) Gel dried out during sample application or incubation.</td>
<td>As for E (iii) above.</td>
</tr>
<tr>
<td>G. Weak, pitted gel</td>
<td>Plate has been frozen.</td>
<td>Repeat using new plate. Review storage.</td>
</tr>
<tr>
<td>H. Poor calibration curve</td>
<td>1. Curve non-linear (Procedure Two)</td>
<td>Incubate for further 24 hours and remeasure the rings.</td>
</tr>
<tr>
<td></td>
<td>(i) Incomplete diffusion.</td>
<td>As for E1 or C1 above.</td>
</tr>
<tr>
<td></td>
<td>(ii) Calibrator rings under/oversize.</td>
<td>(Similar explanations apply to the medium and low calibrator dilutions).</td>
</tr>
<tr>
<td></td>
<td>(iii) Calibration curve constructed incorrectly.</td>
<td>Check calibration curve construction.</td>
</tr>
<tr>
<td>2. y-intercept out-of range (Section 9)</td>
<td>(i) Calibrator rings under/oversize.</td>
<td>As for E1 or C1 above.</td>
</tr>
<tr>
<td></td>
<td>(ii) Calibration curve constructed incorrectly.</td>
<td>(Similar explanations apply to the medium and low calibrator dilutions).</td>
</tr>
</tbody>
</table>

If a problem cannot be resolved, please refer to supplier.
11. EXPECTED VALUES

The following RBP concentrations were obtained using this kit:

<table>
<thead>
<tr>
<th></th>
<th>Mean Conc.</th>
<th>95 Percentile Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg/L)</td>
<td>(mg/L)</td>
</tr>
<tr>
<td>Normal (Males)</td>
<td>52.8</td>
<td>39.0 - 66.0</td>
</tr>
<tr>
<td>Normal (Females)</td>
<td>46.2</td>
<td>32.8 - 60.4</td>
</tr>
</tbody>
</table>

The above results were obtained using normal adult British blood donor serum and are intended for guidance purposes only. Levels can increase more than 3-fold in kidney disease. It is strongly recommended that each user should generate his/her own RBP concentration ranges for appropriate clinical conditions.

12. PERFORMANCE CHARACTERISTICS

A. Precision

The precision (repeatability) of this kit is expressed as the mean and the percentage coefficient of variation (CV) which has been determined using human serum preparations containing high, medium and low concentrations of RBP. All analyses were performed in our laboratory. Each value was calculated from 10 measurements (duplicate determinations on five separate plates from a typical batch) unless otherwise stated. For Procedures ONE and TWO, rings were measured after 96 hours. For Procedure THREE, rings were read after 18 hours.

<table>
<thead>
<tr>
<th>SAMPLE POOL</th>
<th>Procedure ONE Mean Conc. CV</th>
<th>Procedure TWO Mean Conc. CV</th>
<th>Procedure THREE Mean Conc. CV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg/L)</td>
<td>(mg/L)</td>
<td>(mg/L)</td>
</tr>
<tr>
<td>RBP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>86.4</td>
<td>89.2</td>
<td>89.4</td>
</tr>
<tr>
<td>Medium</td>
<td>55.2</td>
<td>55.6</td>
<td>55.4</td>
</tr>
<tr>
<td>Low</td>
<td>22.0</td>
<td>20.2</td>
<td>20.6</td>
</tr>
</tbody>
</table>

B. Within plate and inter-batch variation:

The within plate variation is expressed as the mean ± standard deviation of determinations of CV made using 4 plates from separate batches. Six measurements were made per plate, using a human serum pool as the sample.

The interbatch variation is expressed as the CV of mean concentration values obtained for a human serum pool sample using 4 recent batches of plates. The mean concentration for each batch was determined from six ring measurements per plate, one plate per batch.

<table>
<thead>
<tr>
<th>Within-plate variation</th>
<th>Interbatch variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean CV ± SD</td>
<td>CV (%)</td>
</tr>
<tr>
<td>Procedure ONE</td>
<td>2.14 ± 0.74</td>
</tr>
<tr>
<td>Procedure TWO</td>
<td>2.39 ± 0.65</td>
</tr>
<tr>
<td>Procedure THREE</td>
<td>2.63 ± 0.78</td>
</tr>
</tbody>
</table>
C. Comparison Studies

A correlation study was performed on 115 normal serum samples using this kit and a radial immunodiffusion reference method. The study demonstrated excellent agreement between the two methods, yielding the following linear regression equation and correlation coefficient:

\[ y = 0.998x + 1.145 \]

(y = Binding Site's RBP RID kit)
(x = Reference RBP method)
Correlation coefficient r = 0.975

13. BIBLIOGRAPHY


SUMMARY OF PROCEDURE

1. Select Procedure ONE, TWO or THREE. Procedure THREE must be used if results are required quickly.
2. Reconstitute calibrator and control with the distilled water provided.
3. Prepare calibrator dilution (required for Procedures TWO and THREE).
4. Prepare sample and control dilutions using the BSA provided.
5. Allow condensation to evaporate from RID plate(s).
6. Apply calibrator(s), control and samples to RID plate(s) in 5μL volumes.
7. Replace lid and incubate at room temperature (approximately 20-24°C) for fixed time period (minimum 18 hours) (Procedure THREE) or until rings are complete (minimum 96 hours) (Procedures ONE and TWO).
8. Measure the ring diameters.
9. Read results off RID Reference Table (Procedure ONE) or plot calibration curve and read off results (Procedures TWO and THREE).

THE BINDING SITE LIMITED
PO Box 4073
Birmingham B29 6AT
England
### RID Reference Table for Human ERP 'ML'

**Concentrations in mg/L**

<table>
<thead>
<tr>
<th>Diameter of ring</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0 mm</td>
<td>8.42</td>
</tr>
<tr>
<td>4.1</td>
<td>9.59</td>
</tr>
<tr>
<td>4.2</td>
<td>10.8</td>
</tr>
<tr>
<td>4.3</td>
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**Note:** The above values assume that test samples are applied diluted 1/20 in 5μL volumes.

INSECTS/RIN186
Instruction Manual

Vitamin A/E
by HPLC

Vitamin A/E by HPLC
Reagent Kit, 100 tests
Catalog Number 195-5869

Intended use:
The BIO-RAD Vitamin A/E by HPLC Test is designed for the quantitative determination of Vitamin A and Vitamin E in human serum or plasma.
For in vitro diagnostic use.

Instruction Manual p. 1 - 10
Gebrauchsanweisung S. 11 - 20
Mode d'emploi p. 21 - 30

October 1999
600-0010
Ch. B. 016182
955-910
TECHNICAL ASSISTANCE

Toll Free 1-800-2BIORAD (224-6723)

BIO-RAD provides a toll free line for technical assistance,
available Monday through Friday, 5 am to 5 pm, Pacific Time (PT).

The toll free number is available for use only in the United States of America and Puerto Rico.

Outside the U.S.A., please contact your regional BIO-RAD office for assistance.
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1.0 Introduction

Prematurely born babies since transportation through the placenta had not yet been fully developed and those babies hardly having fatty tissue for storage. A deficiency is indicated by irritability, edemas and hemolytic anemias together with a short lifetime of erythrocytes. A low level of tocopherol may also occur in patients with a malfunctioning absorption of fats or a cystic pancreatitis.

The importance of a simultaneous determination of the Vitamins A and E in serum or plasma has increased considerably since it could be proved that there is a connection between the effect as an absorber of radicals and a protective effect against neoplastic processes. In clinical diagnostics a determination of the Vitamins A and E is used for the diagnosis of pathologic processes caused by insufficient body's defences such as cancer, cardiovascular diseases, alcoholism, cystic fibrosis, inflammations as well as infectious diseases.

Formerly, fat-soluble vitamins in biological material were determined with colorimetric and fluorometric methods, mostly in connection with thin-layer chromatography or open-column chromatography. These methods are very time-consuming, have problems with interfering substances and are relatively insensitive. The development of modern chromatography has considerably improved the analytical methodology for fat-soluble vitamins. Since both vitamins are relatively thermolabile, gas chromatography could only be used in a quite limited degree and thus very soon was replaced by high-performance chromatography (HPLC).

The HPLC-method developed by BIO-RAD allows a fast and precise simultaneous determination of the Vitamins A and E, thus meeting all requirements of laboratory routine.

1.2 Principle

200 μL serum or plasma are mixed with 200 μL of a δ-tocopherol solution. By adding of ammoniumsulfate solution and subsequent centrifugation two phases are developed. The upper phase is used for HPLC Analysis in an isocratic system. The samples are separated on a Reversed Phase Column with subsequent UV-detection and quantitative determination with the help of the Internal Standard.
3.0 Procedure

3.1 Test Procedure

A. Please read carefully the whole manual before performing the test for the first time.

B. Allow all reagents, samples, standards and controls to reach room temperature before this assay is performed. Protect samples, standards and controls from light.

C. A simultaneous analysis of controls is recommended (e.g. BIO-RAD Vitamin A/E Control Set, Level 1 and 2, Cat. No.: 195-5879).

3.2 Sample Collection

For analysis of Vitamin A/E about 500 µL serum or plasma are required. Until analysis samples have to be kept dark and cool (2 – 8 °C) for maximum 12 h. The samples should be stored frozen (< – 18 °C) in the time until analysis persists.

Preparation of standards and controls

3.3.1 Vitamin A/E Serum Standard

The content of the vial is reconstituted in 1 mL dist. and degassed H₂O, standing for 10 min and shake gently to dissolve.

*Stable for 1 week at 2 – 8 °C if stored in the dark.*

3.3.2 Internal Standard

Solve Internal Standard in REAGENT 1 with the relation 1 [g] : 100 [mL] (e.g. 100 mg Internal Standard and 10 mL REAGENT 1).←
Pipette 10 µL of this solution to 10 mL REAGENT 1 and mix well.

*Fresh preparation before every test run is necessary!*

3.3.3 BIO-RAD Controls Cat. No. 195-5879

The content of vial is reconstituted in 1mL dist. and degassed H₂O, standing for 10 min and shake gently to dissolve.

*Stable for 5 days at 2 – 8 °C if stored in the dark.*

Aliquots can be stored frozen for max. 4 weeks at < – 20 °C.
3.0 Procedure

3.6 HPLC Analysis (continued)

3.6.2 First inject the upper-phase of the standard and compare the chromatogram with a former run. Slight shifts of the retention times are mainly a consequence of the age of the separation columns.

3.6.3 Inject the upper-phase of the standard again and use this chromatogram for the calibration.

3.6.4 Inject the upper-phase of the controls and samples

3.6.5 Inject again the upper-phase of the standard immediately after finishing the routine runs. If performing a large run, the upper-phase of the standard should be injected again after 10 - 15 samples.

3.6.6 If the system is used again within three days after finishing of the analysis, it is possible to run it with a flow rate of 0.1 mL/min while the UV-detector is switched off. In case of a longer lasting break it is advisable to take the column out of the system and to store it closed securely. Next the HPLC system should be rinsed with 50 mL HPLC-water followed by 50 mL water/methanol (1/1 v/v).

3.7 System settings:

Pump: flow rate: 2.5 mL/min
Oven: temperature: 45 °C
Detector: Wavelength: 340 nm after 3.0 min: 295 nm
Range: 0.02 after 3.0 min: Range: 0.01

Integrator settings
e.g. HP 3394

Autosampler settings:
e.g. BIO-RAD AS 100

An2: 3
CHT SP: 0.5
PK WD: 0.16
THRSH: 2
AR REJ: 10 000
TIME: 7.5 min
PEAK HEIGHT MODE: 4
Integration Modus: 2

CHROM-LINE settings: Please refer to page 31.

DURATION OF THE CHROMATOGRAM: 7.5 min

* Note:
Please use Mobile Phase for flushing the autosampler.

** Note:
Cooling of the samples within the autosampler results in water and salt precipitation in the bottom of the sample cap. This leads to blockage of the injection valve.
3.0 Procedure

3.10 Calculations (manually without integrator) (continued)

3.10.5 The following Vitamin A concentration is obtained for the samples and controls (Vitamin E concentration is calculated analogue):

\[
\text{Conc. Vit. A (sample) = } \frac{\text{P/H VIT A (sample)}}{\text{P/H IS (sample)}} \times \frac{\text{P/H SE (Std.)}}{\text{P/H VIT A (Std.)}} \times \text{Conc. Std. (ug/ml)***}
\]
4.0 Performance Characteristics

4.2 Linearity

This procedure to determine the Vitamin A resp. Vitamin E concentration has been found to be linear up to 200 µg/dL (Vitamin A) and 8000 µg/dL (Vitamin E).

4.3 Recovery

Patient samples with known Vitamin A resp. Vitamin E concentrations were spiked with a defined amount of Vitamin A resp. Vitamin E. Afterwards the obtained values were standardized with the Internal Standard. The calculated analytical recovery was 97% (Vitamin A) resp. 98% (Vitamin E).

4.4 Reference Ranges

Normal values should be determined by each laboratory to confirm with the characteristics of the population which is being tested.

Recommended reference range:

Vitamin A: 20 – 80 µg/dL serum
Vitamin E: 300 – 1200 µg/dL serum
Introduction

The retinol binding protein enzyme immunoassay (RBP-EIA) was developed as a rapid, inexpensive test for quantification of RBP from individual serum specimens. It is designed for use in laboratories at the provincial- or district-hospital level or by trained epidemiological surveillance teams. Its application allows health care workers to assess the extent of vitamin A deficiency (VAD) within populations. The RBP-EIA is designed to produce data rapidly; to reduce the reliance on costly, centralized laboratory facilities; and to provide an effective tool for field monitoring and recognition of VAD in targeted populations.

Test Principle

The RBP-EIA was developed as an antigen competition assay to detect and quantify RBP from human serum. The test uses purified human RBP adsorbed to microtest strip wells to compete with natural RBP found in serum. To perform the assay, the specimens and control calibrator sera are added to individual wells. A monoclonal anti-RBP antibody, conjugated to horseradish peroxidase (HRP) enzyme, is then immediately added. The test is incubated at room temperature for 15 minutes and then washed. Enzyme substrate is added, incubated for 10 minutes, and the reaction stopped with acid. The test is immediately read on a plate reader, and the results are calculated based on values obtained from the calibration curve. The test results are available in approximately 35 to 40 minutes after the start of the assay.
Currently, the RBP-EIA quantifies RBP from individual serum specimens.

**Materials and Reagents Included in the Kit**

- Five microtest plate covers
- One 96-well, low-protein-binding microtest plate for specimen dilutions
- One resealable pouch containing the 96-well microtiter test plate (12 strip wells) and frame with desiccant
- Two 2-ml vials containing 10 µl of antibody conjugate (concentrated HRP-labeled anti-RBP monoclonal antibody)
- One 0.5-ml vial containing 100 µl of RBP calibrator at 40 µg RBP/ml
- One 30-ml bottle of sample diluent
- One 30-ml bottle of wash buffer, 10x concentration
- One 15-ml bottle of tetramethylbenzidine (TMB) substrate
- One 15-ml bottle of stop solution
- Four sheets of graph paper for plotting results
- Four microtest plate grids for recording specimens and controls
- One instruction booklet

**Additional Laboratory Equipment Needed**

- EIA plate or strip-well reader fitted with a 450-nm filter
- EIA plate or strip-well washer
- deionized (DI) water
- vacuum aspirator
- vortex mixer
- micropipetters and disposable tips
- test tubes
- timepiece or laboratory timer
- laboratory markers
- ¼" hole punch
- 2-ml Eppendorf tubes
- refrigerator (2° to 8°C)
- S&S 903 blood filter paper
- paper towels or similarly absorbent material
Preliminary Operations

1. Warm up kit and samples. All reagents and samples must be at room temperature prior to beginning the assay.
   • Remove the kit from the refrigerator at least 1 hour prior to beginning the assay.
   • Take all reagents, calibrators, and controls out of the box and allow them to warm up on the lab bench for 1 hour.
   • Ensure that all samples are thawed and at room temperature prior to beginning the assay.

2. Dilute the wash concentrate (kit component D) 1:10 with DI water. Accurate dilution is essential for good results.
   • For example, use a 1-liter graduated cylinder to measure 900 ml of DI water and use a 1,000-ml graduated cylinder to measure 100 ml of the wash concentrate (kit component D).
   • Combine the two components in a freshly cleaned plastic container large enough to hold the working wash solution.
   • Stir the working wash solution for at least 5 minutes.
   Note: Never reuse a just-emptied container to hold the working wash solution, as there is a risk of contamination and/or dilution.

Calibrator Preparation

1. Label three small, clean test tubes with a capacity of approximately 1.0 ml as follows:
   Calibrator 1: (40 µg RBP/ml)
   Calibrator 2: (20 µg RBP/ml)
   Calibrator 3: (10 µg RBP/ml)

2. Calculate the dilution of the calibrator required to obtain a final concentration of 40 µg/ml in a 50 µL volume by using the following equations:
   \[(X ÷ 40) \times 50 = V\], where \(X\) = assay value of the provided calibrator, and \(V\) = volume of the provided calibrator.
   \[50 - V = BV\], where: \(BV\) = assay buffer volume required.

3. Prepare the 40 µg/ml calibrator by combining volumes \(V\) and \(BV\) in the test tube labeled Calibrator 1, and mix thoroughly.
4. Pipette 50 µl of the 40 µg/ml calibrator to tube 1.

5. Using a clean pipette tip, add 25 µl of sample diluent to tubes 2 and 3.

6. Transfer 25 µl from tube 1 to tube 2, and mix thoroughly.

7. Using a clean pipette tip, transfer 25 µl from tube 2 to tube 3 and mix thoroughly.

**Sample Preparation: Serum**

1. Using the low-binding microtest plate for dilutions, add 240 µl of sample diluent per well for each specimen and calibrator to be tested.

2. Dilute each specimen and calibrator to be tested 1:25 by adding 10 µl of each to the sample diluent already in the wells.

3. Carefully record the positions of each specimen and the calibrators by their identifier numbers on the plate grid provided.

4. Add diluted specimens to the test strips following the instructions as described in “Performing the Test,” Step 5 (below).

5. At the end of the test procedure, aspirate the remaining volume of the specimen dilutions from the wells of the mixing plate and clearly mark or tape over the used wells to prevent reuse.

**Sample Preparation: Dried Blood Spots (When Validated)**

Note: Follow S&S guidelines for sample collection and handling of DBS.

1. Using a ¼” diameter hole punch, remove one punch from the center of the dried blood spot. Each spot contains 6 µl of serum per ¼” punch.

2. Using forceps, place the ¼” blood spot punch into a tube (Eppendorf 2.0 ml).

3. Add 150 µl of ELISA sample buffer to each ¼” blood spot and vortex.
4. Cap the tube containing the blood spot and buffer and incubate for 18 to 20 hours at 4°C to elute the sera from the filter paper matrix.

5. Post-incubation, vigorously mix each tube containing a sample.

6. Centrifuge each sample at 5,000 rpm for 2 minutes.

7. Remove 100 µl of extract and place into the sample well, as described in “Performing the Test,” Step 5 (below).

8. Record sample ID on plate map and proceed with the rest of the assay.

**Notes**
- If the assay is being performed in duplicate (recommended), two 1⁄4” punches must be eluted per sample. It will also be necessary to double the volume of ELISA sample buffer (to a total of 300 µl) to elute the samples.

- Use a fresh pipette tip for each specimen. Do not reuse any of the wells for dilutions.

- Use all diluted samples, controls, and calibrators within 1 hour of preparation.

**Antibody Conjugate Preparation**
1. Add 990 µl of sample diluent to the amber tube, which contains 10 µl of antibody conjugate.

2. Mix well, and transfer the entire 1.0-ml volume into a clean, dry test tube capable of holding a 15-ml volume.

3. Add 14 ml of sample diluent to the 15-ml tube from above. Mix well.

4. Refrigerate any unused conjugate preparation for use later that same day. Conjugate preparation not used by the end of the test day must be discarded.
Performing the Test

Note: Single-point determinations may be used in this assay. It is strongly recommended, however, that all samples and calibrators be run in duplicate.

1. Open the resealable pouch containing the strip wells and plate frame. Remove as many strips as needed for the number of specimens to be tested. Be sure to include at least 3 wells for the calibrators.

2. Fit the strip wells into the plate frame. Return any unused strip wells to the original pouch for storage and reseal the pouch until they are needed.

3. Vortex the calibrators to mix them just prior to use.

4. Using a fresh pipette tip, transfer 100 µl of each calibrator into the appropriate wells of the test strip. Before aspirating the calibrators, controls, and samples, always pre-wet the pipette tip. Forward or reverse pipette the samples to ensure proper mixing; operators must be consistent with one pipetting technique throughout the assay. Confirm even filling in all tips, and inspect all tips. If leaks are found, do not dispense samples. Replace all tips and try again. If bubbles are found in a pipette tip, do not dispense samples. Replace all tips and try again.

5. Using a fresh pipette tip, transfer 100 µl of each specimen dilution from the dilution plate into the corresponding microtest well of the test strip(s). A multi-channel pipette is recommended (but not required) for this step. Forward or reverse pipette the samples to ensure proper mixing; operators must be consistent with one pipetting technique throughout the assay. As with the calibrator samples, confirm even filling in all tips with no leaks. If leaks are found, do not dispense samples. Replace all tips and try again. If bubbles are found in a pipette tip, do not dispense samples. Replace all tips and try again.

6. Vortex the tube containing the diluted antibody conjugate to mix it, and immediately add 100 µl to each test well. A multi-channel pipette is recommended (but not required) for this step. Confirm even filling in all tips with no leaking tips. If leaks are found, do not dispense samples. Replace all tips and try again. If bubbles are found in a pipette tip, do not dispense samples. Replace all tips and try again. Mix the wells briefly by gently tapping on the strip-well frame.
7. Cover the strip wells with the microtest plate cover and incubate at room
temperature (18° to 25°C) for 15 minutes, mixing briefly at 10 minutes by tapping
the plate frame.

8. Carefully remove the cover and aspirate the wells. Wash the wells 5 successive
times by adding and aspirating wash buffer. After the last wash, tap the plate face
down on a paper towel or absorbent material and blot to remove any remaining
wash buffer.

9. Immediately add 200 µl of TMB substrate to each well, affix a fresh microtest
plate cover, and incubate at room temperature (18° to 25°C) for an additional 10
minutes.

10. Carefully remove the cover, and add 100 µl of stop solution to each well. A multi-
channel pipette is recommended (but not required) for this step. Operators must
be consistent with one pipetting technique throughout the assay. Confirm even
filling in all tips with no leaks. If leaks are found, do not dispense samples.
Replace all tips and try again. If bubbles are found in a pipette tip, do not dis-
pense samples. Replace all tips and try again.

11. Immediately read and record the optical density (OD) of the strip wells in an EIA
plate reader or strip-well reader fitted with a 450-nm filter.

Reading and Interpreting the Results

Using the log graph paper provided:

1. Plot the OD values for the 10, 20, and 40 µg RBP/ml values.

2. Using a ruler, draw a “best fit” between the three points (which should be linear).

3. Plot the OD (Y-axis) readings from the individual specimen tested on the calibra-
tion curve; read the corresponding RBP concentrations (X-axis) expressed in
µg/ml (see Figure B.1).
Appendix B

Figure B.1. Sample Calibration Curve

Note: If duplicate determinations of samples and calibrators are made, use the average of the two OD values for constructing the calibration curve and reading patient and control values.

Assay Validity

An individual RBP-EIA assay is considered valid if the parameters below are observed. Assays that fail to meet these criteria should be considered invalid.

1. The average OD of the 10 µg/ml calibrator is at least 1.200. Assays with lower average OD values should be repeated.

2. The difference in OD (range) between the 10 µg/ml and the 40 µg/ml calibrator is at least 0.650. Assays with lower OD ranges should be repeated.

3. The recommended coefficient of variation (CV) between duplicate OD values for calibrators and samples is < 10%. Specimens with >10% CV should be repeated.

4. The calibrator backfit value (the value obtained from computer programs when the OD of the calibrators is read from the calibration curve) must be within 10% of the calibration value (see Table B.1).
Table B.1. Calibrator Backfit Values

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<th>Valid Backfit Value</th>
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</thead>
<tbody>
<tr>
<td>10 µg/ml</td>
<td>9.0 - 11.0 µg/ml</td>
</tr>
<tr>
<td>20 µg/ml</td>
<td>18.0 - 22.0 µg/ml</td>
</tr>
<tr>
<td>40 µg/ml</td>
<td>36.0 - 44.0 µg/ml</td>
</tr>
</tbody>
</table>

Limitations of the Procedure

- The RBP-EIA kit is for in vitro use only; the results cannot be used as the basis for providing therapy to individual patients.

- Test components from different RBP-EIA kits, and those kit components bearing different lot numbers, must not be mixed or exchanged.

- The incubation times, reagent volumes, dilutions, and incubation temperatures for the RBP-EIA kit have been optimized; any variations or modifications to the test procedure may produce inaccurate results.

- The serum specimens used in the RBP-EIA must be non-hemolyzed and free of any trace of fibrin clots or microbial contamination. Any contaminants may interfere with the performance of the test or accuracy of the results.

- The RBP-EIA most accurately determines RBP concentrations in the 10 to 40 µg RBP/ml region of the calibrator curve. This range of values corresponds to the majority of physiological values for serum RBP, and must be represented in the linear portion of the titration curve. Values established above or below this area may be less accurate.

- The reagents of the RBP-EIA kit must not be used after the expiration date stamped on the kit components and box.
Precautions

- Observe normal laboratory precautions when working with human serum or blood specimens, including the controls.

- The calibrators and controls for this kit are derived from human sera, which were tested and found to be negative for hepatitis B virus surface antigen (HBsAg) and HIV types 1 and 2. However, since no test can provide complete assurance that all infectious agents are absent, these calibrators must be handled as if they were potentially hazardous.

- Some kit reagents contain thimerosal as a preservative. Avoid contact with eyes and skin, and use adequate personal protection while working.
For Additional Information

Safety Precautions


Venipuncture


Sample Handling


Dried Blood Specimens


path

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PATH conducted a series of experiments to determine the feasibility of using dried blood spots (DBS) as specimens for analysis by the RBP-EIA. We conducted experiments in varying the exposure to light, drying conditions, blood volume, hematocrit, and storage temperature to assess the effects on the concentration of RBP. Further to this, we compared the use of whole blood to serum and venous blood to capillary blood (collected by finger prick) on the RBP concentration.

Blood samples were taken from 9 individuals by venipuncture in heparinized (green cap) and serum (red cap) Vacutainer tubes. Heparinized blood from the green cap tubes was spotted onto individual Schleicher & Schuell, Dassel, Germany (S&S) 903 filter paper cards in multiple 90 µl applications for the different tests. Blood collected in the red cap tube was used as a positive control for each patient sample. For capillary blood testing, whole blood was collected directly on filter papers by finger prick.

For the different tests, two 1/4" punches were removed using a standard handheld round hole punch, and were placed into 2.0 ml micro-centrifuge tubes. To each tube, 320 µl of RBP-EIA sample buffer was added, and the tubes were capped and mixed by vortexing. The samples were incubated at 2° to 8°C overnight (approximately 14 to 18 hours) to elute the serum from the DBS. The samples were then exposed to different conditions and assayed with the RBP-EIA to determine the RBP recovery.

The effect of light exposure during drying was evaluated for 8 of the 9 subjects. The RBP concentrations were compared to establish whether exposure to varying intensities of light during sample drying affected the stability of the DBS samples. The results fell within the 10% assay variability as established in the comparative methods (intra- and inter-assay variability) limits.

It was also determined that DBS samples must be dried for a minimum of 2.5 hours for spots dried under conditions of low humidity. In areas of high humidity, it may be necessary to increase the drying time or use a drying chamber with warm circulating air to facilitate drying. We also determined that there is a visual change in the DBS as they dry. After the application of blood, the spots appear wet and bright red; as the spots dry, the color changes to dark brown. Therefore, to ensure that the DBS are completely dry before final storage and to ensure the integrity of the sample, it is
necessary to inspect each sample prior to storage to verify that a color change with drying has taken place.

The effects of blood volume applied to the DBS card on RBP concentration were also considered. We increased and decreased the volume of whole blood (sample) by greater than ±20% during the sample application process, which immediately resulted in a change in the physical size and characteristic of the DBS samples. However, after drying and subsequent elution and assay of the samples at each condition, we found that the volume effects on RBP concentration estimates were well below the inter- and intra-assay variability of the test, e.g. ±10%. Therefore, RBP recovery was shown not to be volume-dependent during the preparation of the DBS.

The fourth test evaluated whether the RBP concentration changed in relation to the hematocrit of the subject’s sample and determined if there is an association between the area of a DBS and hematocrit levels.

In fact, changes in hematocrit did affect the area of the DBS as expected and an inverse relationship between hematocrit levels and the area of the DBS was noted. The RBP levels were found to change slightly, with a 3% to 7% coefficient of variation across the hematocrit percentages tested, but the changes were well within the range of assay variation. That is, differences in the hematocrit levels of whole blood samples did not affect the RBP concentration as determined by RBP-EIA.

Because samples are collected and stored under varying conditions, we considered the effects of storing DBS samples at various temperatures on RBP concentration. Using the –20°C storage condition as a control for each sample, there was little difference in analyte recovery when the test conditions (2° to 8°C, 18° to 25°C, and 45°C) were compared. It should be pointed out that the experiment was only performed for 1 time-point and should be repeated to identify at what point there is degradation in the DBS sample due to storage conditions. There were no changes in RBP concentration after being subjected to the 3 different storage conditions. This may be due in large part to the fact that the samples were completely dried prior to storage and were kept at low humidity by the use of desiccants during storage.

In addition, an experiment was carried out to compare the RBP concentration obtained from DBS prepared from whole blood compared to RBP values obtained from serum samples from the same donor, as this could potentially answer whether whole blood would need to be separated before being analyzed for RBP. There was a significant correlation between the serum and whole blood RBP estimates. The two sample sets correlated within 6%, well within the 10% coefficient of variation necessary for results to be valid, which suggests that there are no significant differ-
ences. Thus, it would appear that DBS samples prepared from whole blood may be suitable for use in the RBP-EIA. However, further experiments and validation studies using a larger subject population with varying degrees of deficiency of VAD will be necessary to give these observations the statistical power to confirm that these premises are valid.

Finally, the RBP levels estimated from DBS samples prepared from capillary finger pricks were compared to RBP values obtained from venous blood from the same donor. This has profound practical implications, as it would potentially remove the need to collect blood by venipuncture. The capillary DBS results averaged within 4.1% of the cumulative serum average. The coefficient of variation was 10.3%, or 0.3% higher than the recommended 10% reproducibility of assay duplicates for valid results. These preliminary data indicated that there was no apparent difference in RBP level between finger-prick and venous DBS. The limited scatter both above and below the serum mean indicated that in this small sampling the relationship between RBP estimates from DBS prepared from venous blood and finger prick specimens was significant.

Further work is planned to validate the use of DBS specimens with the RBP-EIA as this will significantly simplify the field logistics of specimen collection and add to the overall simplicity of the RBP-EIA.