RBP-EIA:

A New Approach to Assessing Vitamin A Deficiency

path
Program for Appropriate Technology in Health

Second Edition
August 2003

About PATH

PATH is an international, nonprofit organization dedicated to improving the health and well-being of people, especially women and children, in the developing world and low-resource settings. Since 1977, PATH has managed more than 1,000 projects in 120 countries.

PATH champions the development, introduction, and adaptation of appropriate and innovative solutions to public health challenges.

For more information about PATH, visit our website at www.path.org.

Acknowledgements

The United States Agency for International Development supported the development of this test and manual under the Technologies for Health (HeathTech) project, Cooperative Agreement Number HRN-A-00-96-90007, managed by PATH.

This manual was produced by PATH's Vitamin A team. We would like to acknowledge Kara Richmond, Todd Alonzo, and Jeff Morgan, who contributed significantly to this document. We are also grateful for the time that Dr. William Blainer, Sherry Tanumihardjo, and Dr. Tianan Jiang spent reviewing this manual.

We would like to offer special thanks to Michele Burns, Jennifer Fox, and NanCee Sautbine for their editing and design work.

RBP-EIA:
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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:

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

Background

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.

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.

Detecting Vitamin A Deficiency: A Public Health Challenge

About This Section

This section provides background information on the challenges inherent to detecting VAD, particularly in low-resource settings. After describing the characteristics of the reference assessment technique, the section introduces retinol binding protein, which is the basis for PATH's RBP-EIA.

Despite the difficulty of estimating the global magnitude of micronutrient malnutrition, there is sufficient information to approximate the number of people affected.[1] The most recent estimates indicate that, worldwide, up to 3.5 billion people are affected with micronutrient malnutrition.[1,2] Of these, more than 100 million are children affected by VAD.[3]

The estimated prevalence of micronutrient deficiencies varies by region.[1] 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.[4] 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.

RBP: A Surrogate Marker for Retinol

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.[4] 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.[5] 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.[6] 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.[6] 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.[6] 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.[7]

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.

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Section 2

Existing Methods for Assessing Vitamin A Status

About This Section

This section provides information on existing methods for assessing vitamin A status. Designed to help researchers and program planners select the most appropriate test for their needs, it summarizes the requirements and performance characteristics of each test.[1]

As discussed in the previous section, the special equipment, training, and handling requirements of HPLC—the refererence 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

Technique	Developer	Analyte Detected	Specimen	Equipment Required	Time Required	Cost	Performance (Sens/Spec)
Tests in Development or Research							
RBP-EIA[2]	PATH	RBP	• Serum	Plate or strip well reader, washer, micropipettes	35-40 minutes per 48 samples; average 400 samples per day	Commercial product not yet available. Cost estimate is US$3.00-$5.00 per determination	Sensitivity: 71% Specificity: 92%
Futterman Fluorescence[3] Assay	Centers for Disease Control and Prevention	Retinol	• Serum • Plasma	Fluorimeter or scanning spectrophotometer, micropipettes	20-40 samples per day	No data available	Method has not been validated for this application
Radio-Immunoassay (RIA)[4,5]	Columbia University	RBP	• Serum • Plasma	Isotope, micro pipettes, vortex mixer, emission counter	No data available	Research method; not commercially available	Sensitivity: 87% Specificity: 98%
Commercially Available Tests							
RBP-EIA[6]	Immunodiagnostik	RBP	• Serum • Plasma • Urine	Plate or strip well reader, washer, incubator, micropipettes	12 hours per 48-96 samples	US$9.00-$18.75 per determination	Research assay; performance not documented
Radial Immunodiffusion Assay (RID)[7]	The Binding Site	RBP	• Serum • Plasma	Micropipettes, jeweler's loupe, passive light source	96 hours per 42 samples	US$4.17 per determination	Not documented
HPLC[8,9,10]	Bio-Rad Craft Technologies	Retinol	• Dried blood spot • Serum • Plasma • Urine • Breast milk	HPLC system, centrifuge, micropipettes, vortex mixer	20-25 samples per day	US$10.00-$25.00 per determination	Reference method; considered gold standard

Additional Information

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.

See Appendix A for the product insert from Immunodiagnostik

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.[8])

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).

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The Technology

About This Section

This section describes the technical platform for PATH's RBP-EIA assay. It provides a brief overview of RBP, how the RBP-EIA is performed, its key characteristics, and answers to frequently asked questions.

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;

- 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.

See Appendix B for the RBP-EIA product insert

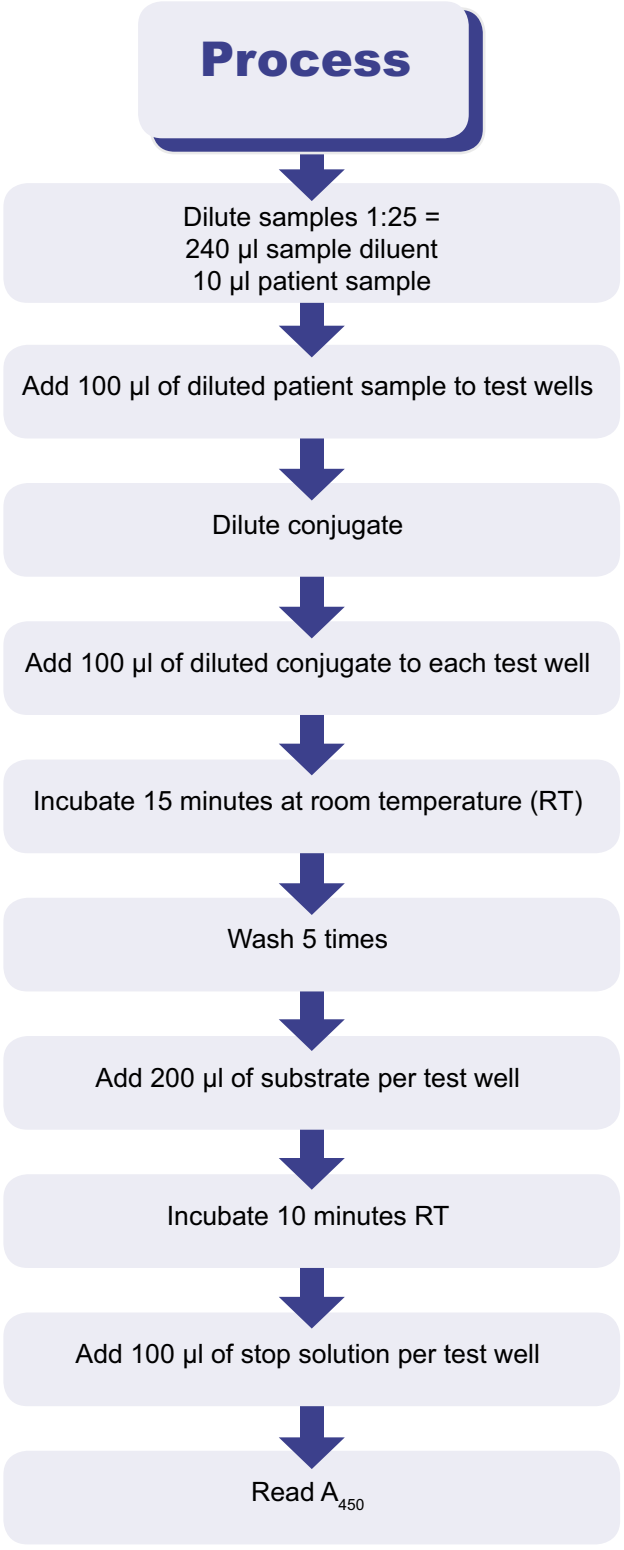
The RBP-EIA is designed to assess and monitor the VAD status in populations. While the results of the assay are quantitative, they should not be considered to be diagnostic of VAD for individuals. Rather, they should be used as a research tool to assess vitamin A status and the extent of VAD in populations.

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



Frequently Asked Questions

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?*

See Appendix B for more information about supply requirements

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 (2º to 8º 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.

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?*

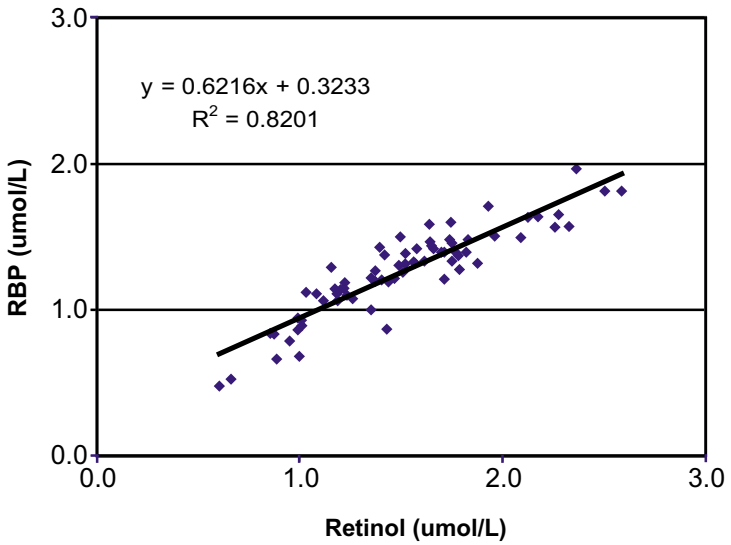
A This cut-off has been established in the literature, and is logical, as retinol and RBP exist in approximately a 1:1 molar ratio.[1]

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



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 populaton 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, ß-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.

See Appendix B for more information

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.[2] 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 RPB 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 37ºC).

References

1. Tietz NW, Pruden EL, McPearson RA, Fuhrman SA. *Clinical Guide to Laboratory Tests.* 3rd ed. Philadelphia: Saunders. pp. 542-543, 634-635 (1995).

2. Gamble MV, Ramakrishnan R, Palafox NA, Briand K, Berglund L, Blaner WS. Retinol binding protein as a surrogate measure for serum retinol: studies in vitamin A-deficient children from the Republic of the Marshall Islands. *Am J of Clin Nutr.* 73:594-601 (2001).

Section 4

Implementation Issues in VAD Assessments

About This Section

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.

See Appendix B for more information on sample handling once in the laboratory, prior to precessing

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
- micropipetters 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.

Section 5

Laboratory and Field Evaluation Results

About This Section

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*[1] 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.

This study was invaluable in that it indicated that assay development was on the right track, and that serum would have to be used to achieve the best correlation.

Establishing Proof of Concept

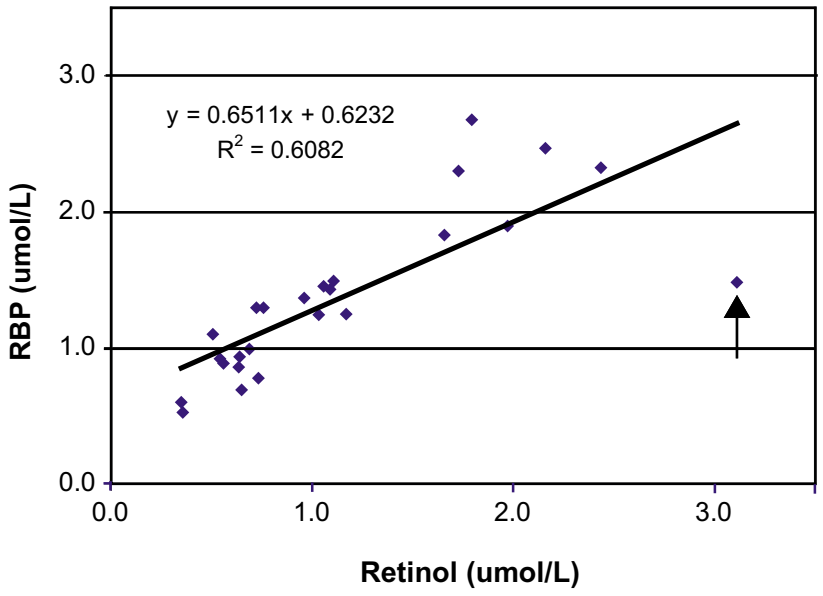
Method

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

Accuracy	0.96 ± 0.04% in the calibrated range*
Inter-assay precision	8.9% CV in the calibrated range
Detection limit	1.1 µg RBP/mL
Quantitation limit	7.7 µg RBP/mL
Linearity	0.997 ± 0.07
Range	.42 to 1.80 µmol RBP/L
Analyte recovery	1.02 ± 0.11
Intra-assay variability	6.7% CV in the calibrated range

*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.*[1] 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.[2]

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).

Table 5.2. Spike Concentrations for RBP-EIA Interference Studies

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

* Test concentrations represent percent increase over normal CRP values.
† Test concentrations represent percent increase over normal RF values.

Prior to assay, each sample was thoroughly mixed in triplicate by two analysts in separate assays, and then stored at 4ºC 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 5.3. Interfering Substances Data

Analyte and Concentration	Assay 1		Assay 2	
	RBP µg/ml	Spike Result T/R	RBP µg/ml	Spike Result T/R
C-reactive protein	21.8	1.04	20.5	0.87
Rheumatoid factor	20.8	1.00	23.9	0.93
Bilirubin, 15 mg/dl	20.5	1.03	18.8	0.92
Hemoglobin, 10 mg/ml	19.8	0.99	20.3	1.00
Human red blood cells, 1%	18.1	0.90	19.5	0.95
Triglycerides, 20 mg/ml	20.7	1.03	19.9	1.02
L-thyroxine, 250 ng/ml	19.1	0.96	20.2	0.99
Vitamin A, 1 µg/ml	20.4	1.02	19.1	0.94
Estrone acetate, 1 µg/ml	19.6	0.98	20.0	0.98
ß-estradiol, 1 µg/ml	20.5	1.03	20.0	0.98
Trans-thyretin, 800 µg/ml	20.4	1.08	20.1	1.03
Reference mean	**19.9**	**1.00**	**19.8**	**0.98**
SD	**0.80**	**0.05**	**0.50**	**0.03**
CV	**4.2%**	**5.1%**	**3.6%**	**3.6%**

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)

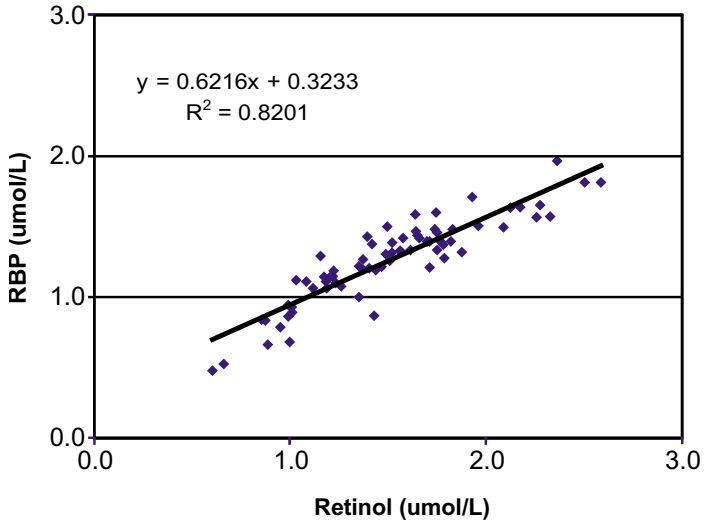
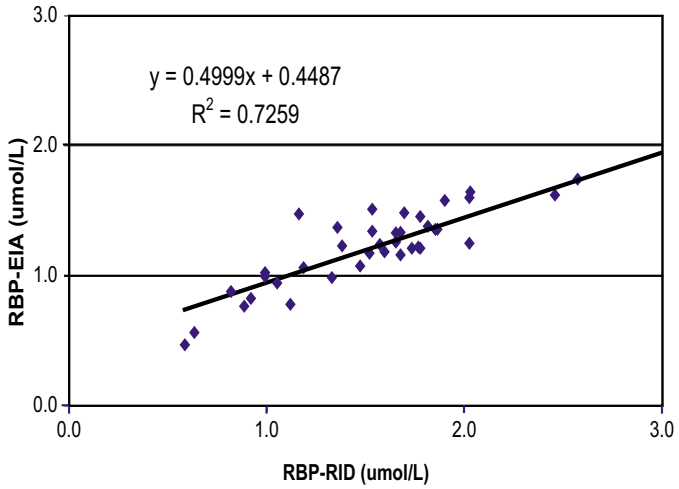


Figure 5.3. Evaluation of RBP-EIA Versus RBP-RID on a Panel of Nicaraguan Sera (n=39)



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 5.4. Distribution of Serum Retinol Levels and RBP Levels (n=359)

VAD level	(%)	95% CI	(%)	95% CI
Severe deficiency (<0.35 µmol/L)	2.2	(0.7, 3.8)	0.6	(0.0, 1.3)
Moderate deficiency (0.35-0.70 µmol/L)	20.1	(15.9, 24.2)	20.3	(16.2, 24.5)
Vitamin A sufficient (<0.70 µmol/L)	77.7	(73.4, 82.0)	79.1	(74.9, 83.3)

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 ≤0.70 µmol/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 µmol/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

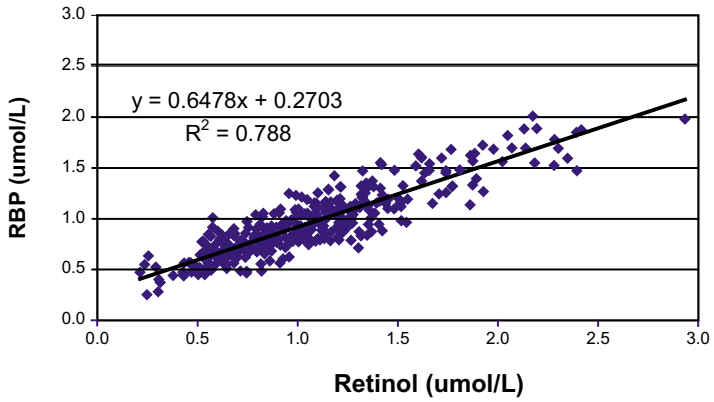
Additional performance characteristics were considered to further assess how the RBP-EIA performed compared to serum retinol by HPLC. A receiver operating characteristic (ROC) curve was produced (Figure 5.5) for RBP concentrations and serum retinol HPLC determinations at different cutoff points. An ROC 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)

	Serum Retinol (≤0.70 µmol/L)		Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
	+	-				
Serum RBP						
<0.70 µmol/L	56	19	70.0% (58.7, 79.7%)	93.2% (89.7, 95.9%)	74.7%	91.5%
≥0.70 µmol/L	24	260				

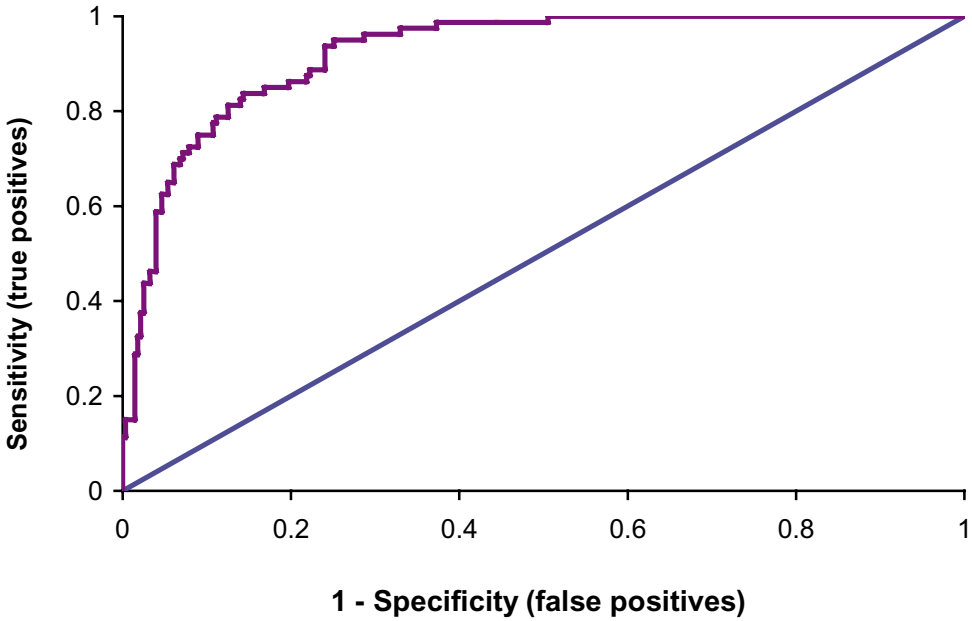
PPV = Positive Predictive Value.
NPV = Negative Predictive Value.

Figure 5.4. Retinol HPLC Determinations Versus RBP-EIA Determinations



The cutoff reflecting VAD at 0.70 µmol/L for both retinol HPLC and RBP-EIA is delineated (n=359).

Figure 5.5. ROC Curve Plotting Relative Sensitivity and Specificity



Area under curve = 0.924, 95% C.I. of area = 0.896 , 0.952)

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 *The United States Pharmacopoeia and The National Formulary,*[1] 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

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RBP-EIA Troubleshooting Guide

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		
General Considerations	**Pipetters**	Pipetters should be calibrated regularly and should be in good working condition. Disposable pipette tips must fit snugly.
	Manual microplate washer	Ensure that all dispenser nozzles are clear and that wash is delivered to wells equally.
	Automated microplate washer	Confirm that washer is programmed appropriately and functioning properly. Ensure that washer is dispensing and aspirating correctly. Adhere to manufacturer's maintenance recommendations.
	Microplate reader	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.
	Specimens	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.
	Specimen temperature	Allow specimens to equilibrate to room temperature (18° to 25°C) prior to testing. Thoroughly mix specimens before testing.
	Reagent temperature	Allow reagents to equilibrate to room temperature (18° to 25°C) prior to testing. Thoroughly mix reagents before use.
	Specimen dilution	Calibrators, controls, and patient specimens are diluted 1:25 in sample diluent. Thoroughly mix diluted specimens.

Initial Troubleshooting: Confirm That Equipment Meets Proper Conditions and Procedures		
General Considerations	**Conjugate reagent preparation**	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.
	Specimen/reagent delivery	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.
	Incubation temperature	Maintain recommended incubation temperature (18° to 25°C) throughout incubation.
	Incubation timing	Adhere to incubation timing outlined in the package insert.
	Wash solution preparation	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.
	Plate washing	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.
	TMB substrate	Use clean disposable containers for TMB delivery. Do not reuse containers. Add TMB substrate within 10 minutes of removal from the reagent bottle.
	Data reduction	Use a linear curve fitting equation.
	Specimen results exceeding	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.

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

Advanced Troubleshooting:
Identify Symptom and Possible Cause

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

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

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

About This Section

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.