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
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immun diagnostik

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 shoul 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

Plasma or serum :	30 - 75	mg/L
Urine :	0,01 - 0,54	mg/L

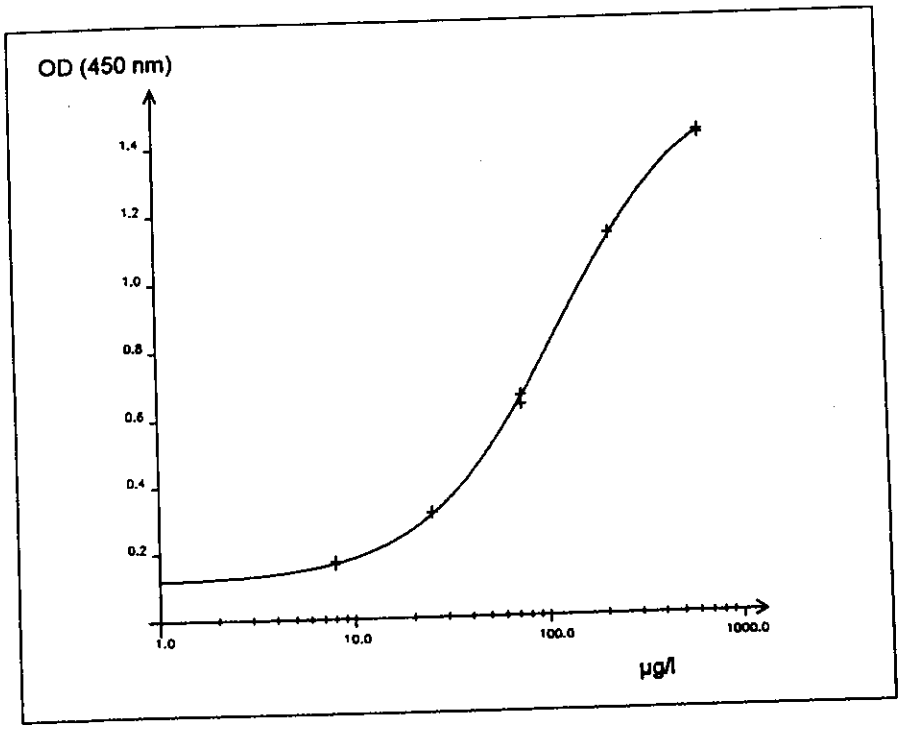
Assay procedure

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. By hand
- 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 (preperation 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:

mean	cv_{within}	$cv_{between}$
[mg/l]	[%]	[%]
0,6	6,8	7,9

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

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NANORID™

SPECIFIC INSTRUCTIONS

THE BINDING SITE

HUMAN RETINOL BINDING PROTEIN 'NL'

NANORID™ RADIAL IMMUNODIFFUSION KIT

For in Vitro Diagnostic Use Only

Product Codes : GD117.3 **Document Code:** RIN186.5 p1 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 Fahey & McKelvey (ref. 4) and Mancini 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 8D). 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. RID 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% E-amino-n-caproic acid (EACA), 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% EACA, 0.01% benzamidine.

C. Control, supplied lyophilised. The expected RBP concentration is given on the vial label. Preservatives:- 0.1% sodium azide, 0.1% EACA, 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. RID plates should be stored at 2-8°C and are damaged by temperature extremes. Freezing will destroy the gel, therefore RID 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 aliquoted 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 BSA 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 GD117.3

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 7% 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 5µL volumes. Binding Site 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) <u>RID Plate(s)</u>

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 measuring 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 resealed in their foil pouches and stored at 2-8°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%, ie to 6 parts in 10) and a low dilution (10%, ie 1 part in 10). It is recommended that 120µL of calibrator is mixed with 80µL of the diluent provided (7% BSA) for a 60% dilution, and 25µL of calibrator is mixed with 225µL 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 10µL of test sample is mixed with 190µL 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 10µL of test sample is mixed with 190µL of diluent (7% BSA). Mix gently before use. If samples containing high RBP 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 10µL of test sample is mixed with the appropriate volume of BSA. For samples having RBP 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 neat 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 5µL 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 5µL 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 96 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 10% 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 51mg/L, 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 jewellers' 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 (ie. 1/20) must be taken into account when calculating the results.

Example

Test Sample	Dilution	Ring Diameter (mm)	Table Value (mg/L)	Original Sample concn. (mg/L)
RBP Serum	1/20	6.8	51.0	51.0
RBP Serum	1/20	>11	>156	>156
RBP Serum (Repeat)	1/40	8.6	90.1	180.2*

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

Procedure TWO.

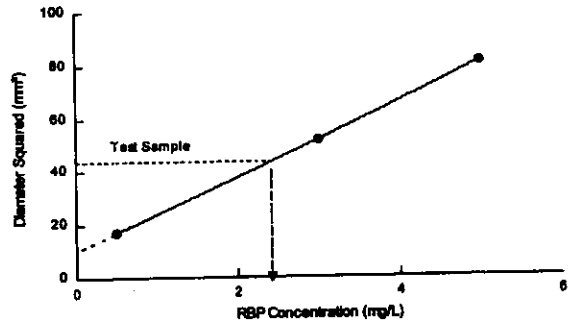
Plot the square of the diameters of the precipitin rings formed by the neat calibrator plus the two dilutions versus their concentrations (given on the neat calibrator vial label). RBP concentrations should be along the horizontal (x) axis, ring diameters squared along the vertical (y) axis. A line of best of fit is drawn through the three points; the y-intercept should be in the range 10-12mm^2. 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 (ie the neat calibrator plus the two dilutions) gave the following ring diameters on a RBP test plate at completion:

Calibrator	Concn. (mg/L)	Diameter (D) of ring (mm)	D squared (mm^2)
Neat	5.0	9.0	81.0
60%	3.0	7.2	51.8
10%	0.5	4.1	16.8

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.

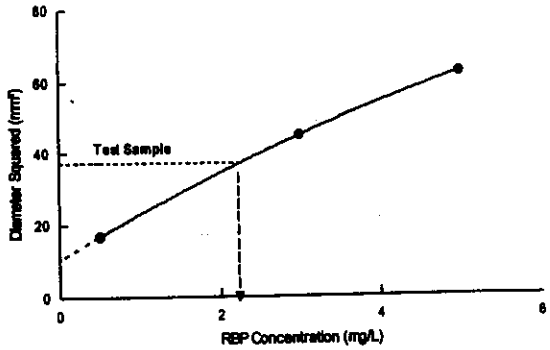
Procedure THREE

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

Calibrator	Conc. (mg/L)	Diameter (D) of ring (mm)	D squared (mm^2)
Neat	5.0	7.9	62.4
60%	3.0	6.7	44.9
10%	0.5	4.1	16.8



An unknown sample, applied diluted 1/20 as recommended, gave a 6.1mm ring on this plate. From the above curve, this corresponds to a RBP concentration of 2.32mg/L. Therefore, the RBP concentration in the undiluted sample = 2.32 x 20 = 46.4mg/L.

10. **LIMITATIONS OF PROCEDURES**

A. For Procedure ONE, results generated from ring diameters greater than the neat calibrator ring diameter (ie 9mm) should be regarded as approximate (See Section 9). For procedures TWO and THREE, accurate results are limited to the calibration curve between the neat 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**

	Problem	Possible Causes(s)	Suggested Action(s)
A.	<u>No ring for:</u>		
1.	Calibrator(s)	Calibrator omitted.	Repeat assay.
2	Test sample.	(i) Sample omitted. (ii) Concentration too high/low.	Repeat assay. Dilute/concentrate and reassay.
3.	Calibrator(s) and test samples.	Plate deterioration.	a) Storage damage. Repeat assay using new plate. b) Product expired. Repeat assay using new plate/kit.
B.	<u>Oversize Rings for:</u>		
1.	Neat calibrator (more than 9.3mm)	(i) Inaccurate ring measurement. (ii) Incorrect volume applied. (iii) Inaccurate volume applied.	Remeasure using eyepiece or RID Plate Reader. Check 5µL 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.
		(v) 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.
		(vii) Local antibody depletion due to adjacent high concentration test samples.	Dilute the sample(s) responsible and repeat assay using new plate.
		(viii) Incubation temperature too high (see Section 8F).	Repeat assay, incubating at 22°C.
2.	Test samples (above acceptable range - see Section 10)	(i) Concentration too high.	Dilute and reassay.
		(ii) Incorrect volumes applied.	Check 5µL volume applied.
C.	<u>Undersized rings for</u>: 1. Neat calibrator (less than 8.7mm)	(i) Inaccurate ring measurement. (ii) Incorrect volume applied. (iii) Inaccurate volume applied. (iv) Inaccurate calibrator reconstitution	As for B1 above.
		(v) Calibrator deterioration.	(a) Storage damage. Repeat assay using new calibrator. (b) Product expired. Repeat assay using new kit.
		(vi) Incubation temperature too low (see Section 8F).	Repeat assay, incubating at 22°C.
2.	Test samples (below acceptable range - see Section 10A).	(i) Concentration too low.	See Section 8C(4) and repeat assay.
		(ii) Incorrect volume applied.	Check 5µL applied.
D.	<u>Double/Multiple rings</u>	(i) Non-specific precipitation close to well (due to PEG in gel).	Read outer ring.
		(ii) Poor sample application.	Repeat assay.
		(iii) Calibrator deterioration	(a) Storage damage. Repeat assay using new calibrator. (b) Product expired. Repeat assay using new kit.

	Problem	Possible Cause(s)	Suggested Action
		(iv) Sample deterioration.	Reassay using fresh sample.
E.	Non-circular rings	(i) Poor sample application.	Repeat assay.
		(ii) Gel dried out before use.	(a) Storage damage. Repeat assay using new plate. (b) Product expired. Repeat assay using new plate/kit.
		(iii) Gel dried out during sample application or incubation.	Repeat assay minimising the time the plate is left open. Incubate with lid on tight in a moist box or sealed foil pouch.
		(iv) Local antibody depletion (due to high concentration samples on the plate.)	Dilute samples and repeat assay.
F.	Cloudy gel	(i) Plate has been frozen.	Repeat assay using new plates. Review storage.
		(ii) Gel dried before use.	As for E (ii) above.
		(iii) Gel dried out during sample application or incubation.	As for E (iii) above.
G.	Weak, pitted gel	Plate has been frozen.	Repeat using new plate. Review storage.
H	Poor calibration curve		
1.	Curve non-linear (Procedure Two)	(i) Incomplete diffusion.	Incubate for further 24 hours and remeasure the rings.
		(ii) Calibrator rings under/oversize.	As for B1 or C1 above. (Similar explanations apply to the medium and low calibrator dilutions).
		(iii) Calibration curve constructed incorrectly.	Check calibration curve construction.
2.	y-intercept out-of range (Section 9)	(i) Calibrator rings under/oversize.	As for B1 or C1 above. (Similar explanations apply to the medium and low calibrator dilutions).
		(ii) Calibration curve constructed incorrectly.	Check calibration curve construction.

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

11. EXPECTED VALUES

The following RBP concentrations were obtained using this kit:

		Mean Concn. (mg/L)	95 Percentile Range (mg/L)
Normal (Males)	(N=47)	52.8	39.0 - 66.8
Normal (Females)	(N=55)	46.2	32.8 - 60.4

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.

SAMPLE POOL		Procedure ONE Mean Conc. (mg/L)	CV	Procedure TWO Mean Conc. (mg/L)	CV	Procedure THREE Mean Conc. (mg/L)	CV
RBP	High	86.4	2.3%	89.2	2.5%	89.4	4.3%
	Medium	55.2	2.7%	55.6	2.9%	55.4	2.8%
	Low	22.0	3.4%	20.2	3.7%	20.6	4.1%

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.

	Within-plate variation Mean CV% ± SD	Interbatch variation CV (%)
Procedure ONE	2.14 ± 0.74	2.55
Procedure TWO	2.39 ± 0.65	1.37
Procedure THREE	2.63 ± 0.78	3.18

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 :

$y = 0.998x + 1.165$
(y = Binding Site's RBP RID kit)
(x = Reference RBP method)
Correlation coefficient $r = 0.975$

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2. Haider, M and Haider, S Q (1984). Assessment of protein-calorie malnutrition. Clin. Chem. 30, 1286-1299.

3. Ayatse, J O I and Kwan, J T C (1991). Relative sensitivity of serum and urinary retinol binding protein and alpha-1 microglobulin in the assessment of renal function. Ann. Clin. Biochem. 28, 514-516.

4. Fahey, J L & McKelvey, E M (1965). Quantitative determination of serum immunoglobulins in antibody-agar plates. J. Immunol,. 94, 84-90.

5. Mancini, G, Vaerman, J P et al. (1964). Protides of the biological fluids (XI Colloquium). Peters H. (ed), Amsterdam, Elsevier Publishing Co., p 370.

6. Mancini, G, Carbonara, A O et al (1965). Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochem. 2, 235-254.

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 RBP 'NL'
Concentrations in mg/L

Diameter of ring	Conc.
4.0mm	8.45
4.1	9.59
4.2	10.8
4.3	12.0
4.4	13.2
4.5	14.4
4.6	15.7
4.7	17.0
4.8	18.4
4.9	19.7
5.0	21.1
5.1	22.5
5.2	24.0
5.3	25.5
5.4	27.0
5.5	28.5
5.6	30.1
5.7	31.7
5.8	33.3
5.9	34.8
6.0	36.6
6.1	38.3
6.2	40.1
6.3	41.8
6.4	43.6
6.5	45.4
6.6	47.3
6.7	49.1
6.8	51.0
6.9	53.0
7.0	54.9
7.1	56.8
7.2	58.9
7.3	61.0
7.4	63.0
7.5	65.1
7.6	67.3
7.7	69.4
7.8	71.6
7.9	73.8
8.0	76.1
8.1	78.3
8.2	80.6
8.3	82.9
8.4	85.3
8.5	87.7
8.6	90.1
8.7	92.5
8.8	95.0
8.9	97.5
9.0	100
9.1	103
9.2	105
9.3	108
9.4	110
9.5	113
9.6	116
9.7	118
9.8	121
9.9	124
10.0	127
10.1	130
10.2	132
10.3	135
10.4	138
10.5	141
10.6	144
10.7	147
10.8	150
10.9	153
11.0	156

Note: The above values assume that test samples are applied diluted 1/20 in 5µL volumes.

INSERTS/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.

October 1999
600-0010
Ch. B. 018182
955:910

BIO-RAD

Vitamin A/E by HPLC

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TECHNICAL ASSISTANCE

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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.

Contents

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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 quanitative 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

Caution:
All patient samples should be considered potentially biohazardous and should be handled with caution.

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 reconsituted in 1 mL dist. and degassed H_2O, 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 if necessary!

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

The content of vial is reconstituted in 1mL dist. and degassed H_2O, standing for 10 min and shake gently to dissolve.
Stable for 5 days at 2 – 8 °C if stored in the dark.

Aliquotes 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).

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

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

Att2^: 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

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

Sample loop: 20 µL
Filling Speed: 500 µL/min
Temperature: 15–20 °C**
Overfill: 40 µL
Time: 8.5 minutes
Flush volume: 1 mL

*** 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.

CHROM-LINE settings: Please refer to page 31.

DURATION OF THE CHROMATOGRAM: 7.5 min

3.0 Procedure

3.10 Calculations (manually without integrator) (continued)

**** Attention:*
Vitamin A resp. Vitamin E concentration (reconstituted Standard): Please refer to the attached insert.

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

$$\text{Conc. Vit. A (sample)} = \frac{\text{PH Vit. A (sample)}}{\text{PH IS (sample)}} \times \frac{\text{PH IS (Std.)}}{\text{PH Vit. A (Std.)}} \times \text{Conc. Std. [µg/dl]}^{***}$$

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

Appendix B

RBP-EIA Product Insert

Introduction

The retinol binding protein ezyme 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.

Processing Specimens With the RBP-EIA

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:

   $(X \div 40) \times 50 = V$, 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 ¼" 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 dispense 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

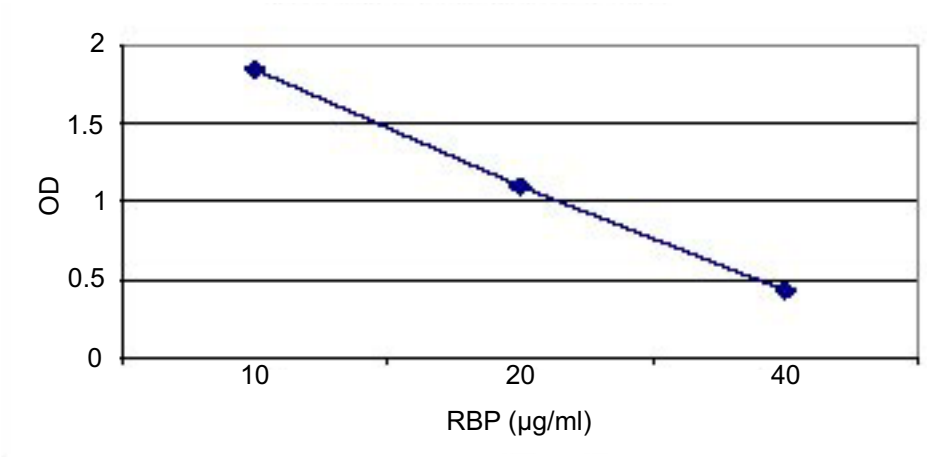
Using the log graph paper provided:

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 calibration curve; read the corresponding RBP concentrations (X-axis) expressed in µg/ml (see Figure B.1).

Figure B.1. Sample Callibration 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

Calibrator	Valid Backfit Value
10 µg/ml	9.0 - 11.0 µg/ml
20 µg/ml	18.0 - 22.0 µg/ml
40 µg/ml	36.0 - 44.0 µg/ml

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

Department of Labor, Department of Health and Human Services. *Joint Advisory Notice: Protection Against Occupational Exposure to Hepatitis B Virus (HBV) and Human Immunodeficiency Virus (HIV)*. Washington, D.C.: US Department of Labor, US Department of Health and Human Services (1987).

Garner JS, Simmons BP. Guidelines for isolation precautions in hospitals. *Infect Control*. 4:245-325 (1983).

Immunization Practices Advisory Committee. Recommendations for protection against viral hepatitis. *MMWR*. 34:313-324, 329-335 (1985).

Venipuncture

National Committee for Clinical Laboratory Standards. *NCCLS Approved Standard LA4-A2*. http://www.upstate.edu/phlebotomy/pages/venipunc/venitec3.htm.

Sample Handling

Villanova PA. *Blood Collection on Filter Paper for Neonatal Screening Programs. National Committee for Laboratory Standards* (June 1992). Available online at http://www.upstate.edu/phlebotomy/index.html.

Dried Blood Specimens

Guidelines for the Shipment of Dried Blood Spot Specimens. Available online at http://www.cdc.gov/od/ohs/biosfty/driblood.htm.

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Appendix C

Use of Dried Blood Spots as Specimens for the RBP-EIA

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 ¼" 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.