Version 23-01/25

User's Manual

Retinol Binding Protein (RBP) ELISA Kit

REF **DEIASL549**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

The Retinol Binding Protein (RBP) kit is designed to quantitatively measure RBP present in serum, plasma, dried blood spot (DBS) and urine samples. Please read the complete kit insert before performing this assay.

General Description

Retinol binding protein (RBP) is from a family of structurally related proteins that bind small hydrophobic molecules such as bile pigments, steroids, odorants, etc1 . RBP is a 21 kDa highly conserved, single-chain glycoprotein, consisting of 182 amino acids with 3 disulfide bonds, that has a hydrophobic pocket which binds retinol (vitamin A).

RBP binds retinol in a 1:1 stoichiometry, which serves to not only solubilize retinol but also protect it from oxidation. When in serum, the majority of RBP bound with retinol is reversibly complexed with transthyretin (prealbumin)2,3. This complex then transports retinol to specific receptors of various tissues in the body. Vitamin A status is reflected by serum concentration as it is hemostatically controlled and does not fall until stores are dramatically reduced4,5.

RBP has been shown to be a useful surrogate marker for retinol because of the approximate 1:1 (molar) correlation between retinol and RBP in serum1, 6,7, which implies that RBP may be used to assess and monitor vitamin A deficiency (VAD) in populations. The World Health Organization has estimated that 250 million children have moderate to severe VAD7 due to lack of adequate nutrition, and the rising cost of food staples around the world further exacerbates this problem. In addition to nutritional deficiencies, infectious stresses have been shown to depress retinol concentrations.

RBP has also been shown to be a useful marker for renal function8 as it is totally filtered by the glomeruli and reabsorbed by proximal tubules9 . This has made the measurement of urinary RBP a tool to study renal function in heart10 or kidney11 transplant recipients.

Principles of Testing

The kit offers two standard curve ranges. For serum and plasma samples, we recommend using 10 µL of standards and samples with an assay range of 1,000 to 7.813 ng/mL. For urine and dried blood spot samples, we recommend using 100 µL of standards and samples with an assay range of 200 to 1.563 ng/mL.

A RBP standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve. Standards or diluted samples are pipetted into a clear microtiter plate coated with an antibody to capture sheep antibodies. A RBP-peroxidase conjugate is added to the standards and samples in the wells. The binding reaction is initiated by the addition of the RBP polyclonal antibody to each well. After an hour incubation the plate is washed and substrate is added. The substrate reacts with the bound RBP-peroxidase conjugate. After a short incubation, the reaction is stopped and the intensity of the generated color is detected in a microtiter plate reader capable of measuring 450 nm wavelength. The concentration of RBP in the sample is calculated, after making a suitable correction for the dilution of the sample, using software available with most plate readers.

Reagents And Materials Provided

1. Coated Clear 96 Well Plates (1 Each)

A clear plastic microplate(s) with break-apart strips coated with donkey anti-sheep IgG.

2. RBP Standard (60 µL)

A stock solution of native human RBP at 20 µg/mL.

3. RBP Antibody (3 mL)

A polyclonal antibody specific for RBP.

4. Conjugate (3 mL)

A RBP-peroxidase conjugate.

5. Assay Buffer Concentrate (8 mL)

A 5X concentrate that must be diluted with deionized or distilled water.

6. Wash Buffer Concentrate (30 mL)

A 20X concentrate that must be diluted with deionized or distilled water.

7. TMB Substrate (11 mL)
8. Stop Solution (5 mL)

A 1M solution of hydrochloric acid. CAUSTIC.

9. Plate Sealer (1 Each)

Materials Required But Not Supplied

1. Distilled or deionized water.
2. A microplate shaker and a microplate washer.
3. Repeater pipet with disposable tips capable of dispensing 25 µL, 50 µL, and 100 µL.
4. Colorimetric 96 well microplate reader capable of reading optical density at 450 nm.
5. Software for converting raw relative optical density readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

Storage

All components of this kit should be stored at 4°C until the expiration date of the kit.

Specimen Collection And Preparation

This assay has been fully validated for human serum, plasma, urine and dried blood spot samples. Samples containing visible particulate should be centrifuged prior to using.

Serum and Plasma

10 µL Format Serum and plasma samples must be diluted 1:40 by taking one part of serum or plasma and adding thirty-nine parts of diluted Assay Buffer prior to running in the kit.

Urine Samples

100 µL Format Urine samples must be diluted 1:4 by adding one part of urine to three parts diluted Assay Buffer prior to running in the kit. Any samples with RBP concentrations greater than the standard curve range should be diluted further with diluted Assay Buffer to obtain readings within the standard curve.

Dried Blood Spots (DBS)

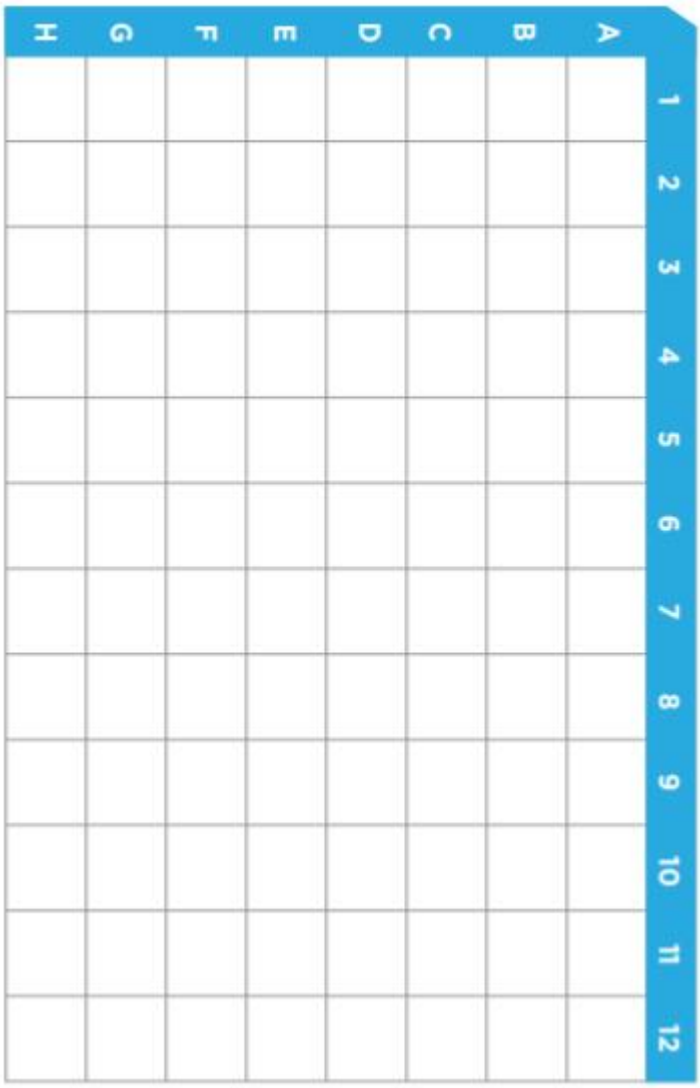
100 µL Format Dried blood spot (DBS) samples should be prepared according to the 2007 Clinical Chemistry paper by Masako Fujita, et al, vol. 53 (11), page 1972-1975. Briefly, whole blood is spotted onto Whatman 309 filter paper and thoroughly dried at room temperature. These can be stored desiccated at ≤ 4°C until use. DBS samples, 1/4" or 1/8", are punched out into clean plastic tubes with caps. The DBS samples require ≥ 1:60 dilution in diluted Assay Buffer.

One 1/4" DBS sample is equivalent to 6 µL of a whole blood sample and we recommend adding 900 µL diluted Assay Buffer. This is a dilution of 1:150. Two 1/8" DBS will contain the equivalent to 3 µL of whole blood sample and we recommend adding 900 µL diluted Assay Buffer. This is a dilution of 1:300. The tubes are capped and left at 4°C overnight. The following morning, the red solution can be run without centrifugation or further dilution.

For calculation purposes a 1/4 inch DBS is considered to contain 6 µL of whole blood and a 1/8 inch DBS is considered to contain 1.5 µL of whole blood sample. The dilution of any samples that fall outside the standard range should be adjusted to allow samples to read within the standard curve.

Use all samples shortly after dilution.

Plate Preparation



Reagent Preparation

Allow the kit reagents to come to room temperature for 30 minutes. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Assay Buffer

Dilute Assay Buffer Concentrate 1:5 by adding one part of the concentrate to four parts of deionized water. Once diluted this is stable for 3 months at 4°C.

Wash Buffer

Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted this is stable at room temperature for 3 months.

Standard Preparation

Standard Preparation - 10 µL Assay Format - Serum and Plasma Samples

Label test tubes as #1 through #8. Pipet 190 µL of Assay Buffer into tube #1 and 50 µL into tubes #2 to #8.

Carefully add 10 µL of the RBP stock solution to tube #1 and vortex completely. Take 50 µL of the RBP solution in tube #1 and add it to tube #2 and vortex completely. Repeat the serial dilutions for tubes #3 through #8. The concentration of RBP in tubes 1 through 8 will be 1,000, 500, 250, 125, 62.5, 31.25, 15.625, and 7.813 ng/mL.

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8
Assay Buffer (µL)	**190**	50	50	50	50	50	50	50
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
Vol of Addition (µL)	**10**	50	50	50	50	50	50	50
Final Conc (ng/mL)	1,000	500	250	125	62.5	31.25	15.625	7.813

Standard Preparation - 100 µL Assay Format - Urine and Dried Blood Spot Samples

Label test tubes as #1 through #8. Pipet 990 µL of Assay Buffer into tube #1 and 300 µL into tubes #2 to #8. Carefully add 10 µL of the RBP stock solution to tube #1 and vortex completely. Take 300 µL of the RBP solution in tube #1 and add it to tube #2 and vortex completely. Repeat the serial dilutions for tubes #3 through #8. The concentration of RBP in tubes 1 through 8 will be 200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.563 ng/mL.

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8
Assay Buffer (µL)	**990**	300	300	300	300	300	300	300
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
Vol of Addition (µL)	**10**	300	300	300	300	300	300	300
Final Conc (ng/mL)	200	100	50	25	12.5	6.25	3.125	1.563

Use all Standards within 2 hours of preparation.

Assay Procedure

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine RBP concentrations.

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification. Determine the number of wells to be used and return unused wells to the foil pouch with desiccant. Seal the ziploc plate bag and store at 4˚C.
2. Pipet 10 µL (100 µL for alternate format) of samples or standards into wells in the plate.
3. Pipet 35 µL (125 µL for alternate format) of Assay Buffer into the non-specific binding (NSB) wells.
4. Pipet 10 µL (100 µL for alternate format) of Assay Buffer into the maximum binding (B0 or Zero standard) wells.
5. Add 25 µL of the RBP Conjugate to each well using a repeater pipet.
6. Add 25 µL of the RBP Antibody to each well, except the NSB wells, using a repeater pipet.
7. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 1 hour. We recommend shaking at around 700–900 rpm. If the plate is not shaken, signals bound will be approximately 15% lower.
8. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.
9. Add 100 µL of the TMB Substrate to each well, using a repeater pipet.
10. Incubate the plate at room temperature for 30 minutes without shaking.
11. Add 50 µL of the Stop Solution to each well, using a repeater or a multichannel pipet.

12. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.
13. Use the plate reader's built-in 4PLC software capabilities to calculate RBP concentration for each sample.

NOTE: If you are using only part of a strip well plate, at the end of the assay throw away the used wells and retain the plate frame for use with the remaining unused wells.

Calculation

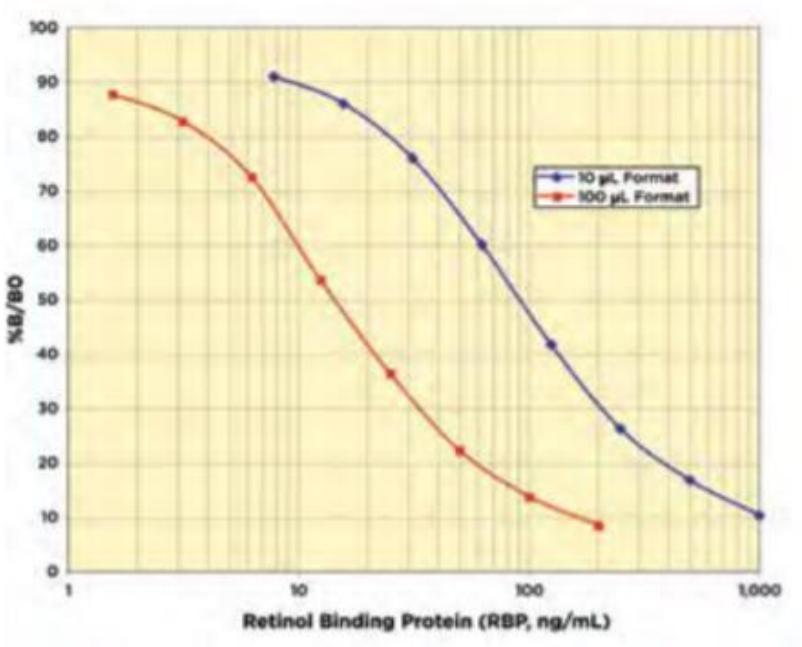
Average the duplicate OD readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean OD's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values

Typical Standard Curve

Always run your own standard curve for calculation of results. Do not use this data.

	10 µL Assay				100 µL Assay			
Sample	Mean OD	Net OD	% B/B0	RBP Conc. (ng/mL)	Mean OD	Net OD	% B/B0	RBP Conc. (ng/mL)
NSB	0.066	0.000	-	-	0.064	0.000		-
Standard 1	0.234	0.168	10.3	1,000	0.137	0.073	8.64	200
Standard 2	0.338	0.272	16.7	500	0.182	0.118	14.0	100
Standard 3	0.492	0.426	26.2	250	0.254	0.190	22.5	50
Standard 4	0.744	0.678	41.7	125	0.373	0.309	36.6	25
Standard 5	1.043	0.977	60.2	62.5	0.518	0.454	53.7	12.5
Standard 6	1.300	1.234	76.0	31.25	0.678	0.614	72.7	6.25
Standard 7	1.463	1.397	86.0	15.625	0.764	0.700	82.8	3.125
Standard 8	1.544	1.478	91.0	7.813	0.806	0.742	87.8	1.563
B0	1.690	1.624	100	0	0.909	0.845	100	0
Sample 1	0.778	0.712	43.8	116.2	0.337	0.273	32.3	29.7
Sample 2	1.131	1.065	65.6	50.45	0.580	0.516	61.1	7.22

Conversion Factor: 1 ng/mL of human RBP is equivalent to 47.62 pM RBP.



Reference Values

Eleven random human urine samples were tested in the assay. Values adjusted for dilution ranged from 7.13 to 98.66 ng/mL with a mean of 32.27 ng/mL.

Ten normal human serum samples were tested in the assay. Values adjusted for dilution ranged from 23.1 to 45.4 µg/mL with a mean of 32.38 µg/mL. Eleven normal human plasma samples were tested in the assay. Adjusted values ranged from 18.13 to 49.8 µg/mL with a mean of 26.70 µg/mL.

Twenty random human whole blood DBS samples were punched out as 1/4" or 1/8" and tested in the assay. Adjusted values ranged from 9.80 to 33.67 µg/mL with an average of 18.8 µg/mL.

Precision

Intra Assay Precision - 10 µL Format

Three human serum and plasma samples were diluted with Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated RBP concentrations were:

Sample	RBP Conc. (ng/mL)	%CV
1	52.6	6.1
2	120.8	4.8
3	182.6	3.7

Inter Assay Precision - 10 µL Format

Three human serum and plasma samples were diluted with Assay Buffer and run in duplicates in twenty-three assays run over multiple days by four operators. The mean and precision of the calculated RBP concentrations were:

Sample	RBP Conc. (ng/mL)	%CV
1	45.3	16.0
2	112.0	11.6
3	174.6	9.3

Inter Assay Precision - 100 µL Format

Three human serum and plasma samples were diluted with Assay Buffer and run in duplicates in ten assays run over multiple days by four operators. The mean and precision of the calculated RBP concentrations were:

Sample	RBP Conc. (ng/mL)	%CV
1	8.2	11.4
2	19.9	9.3
3	31.5	9.9

Detection Limit

The Limit of Detection for the 10 µL and the 100 µL sample format was determined in a similar manner by comparing the OD's for twenty runs for each of the zero standard and a low concentration human sample in the appropriate volume standard curve.

Limit of Detection was determined as 8.09 ng/mL for 10 µL and 0.995 ng/mL for 100 µL sample size.

Sensitivity

Sensitivity with the 10 µL and the 100 µL sample volume was calculated by comparing the OD's for twenty wells run for each of the B0 and standard #8. The detection limit was determined at two (2) standard deviations from the B0 along the appropriate volume standard curve.

Sensitivity was determined as 5.69 ng/mL for 10 µL and 1.36 ng/mL for 100 µL sample size.

Specificity

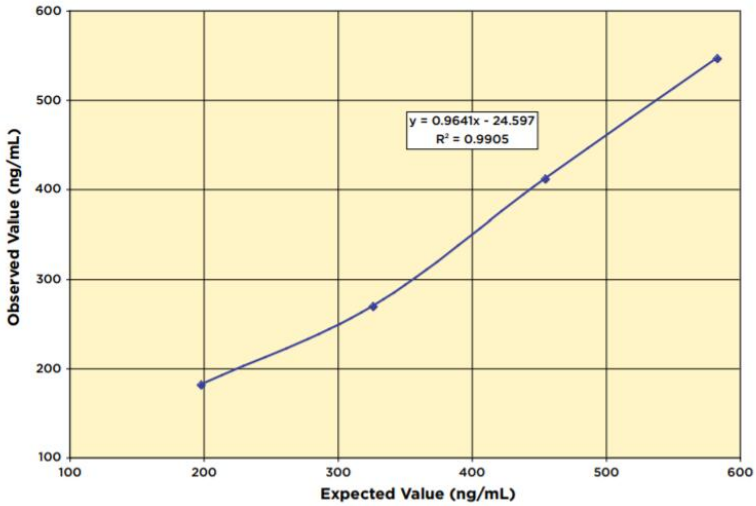
The following cross reactant was tested in the assay and calculated at the 50% binding point.

Analyte	Cross Reactivity (%)
RBP4	23.2%

Linearity

Linearity was determined for the 10 µL format using human plasma samples, by taking samples with a high known RBP concentration and a lower RBP concentration and mixing them in the ratios given below. The measured RBP concentrations were compared to the expected values based on the ratios used.

High Sample	Low sample	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
80%	20%	583.1	546.2	93.7%
60%	40%	454.8	411.4	90.5%
40%	60%	326.4	269.2	82.5%
20%	80%	198.1	181.1	91.4%
			Mean Recovery	**89.5%**



Precautions

1. As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.
2. The antibody coated plate needs to be stored desiccated. The silica gel pack included in the foil ziploc bag will keep the plate dry. The silica gel pack will turn from blue to pink if the ziploc has not been closed properly.
3. The RBP Standard is purified from a human source and as such, should be treated as potentially hazardous. Proper safety procedures must be followed.
4. This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturers Wash Buffers, containing sodium azide will inhibit color production from the enzyme. Make sure all buffers used for samples are azide free. Ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer as prepared on "Reagent Preparation".
5. The Stop Solution is acid. The solution should not come in contact with skin or eyes. Take appropriate precautions when handling this reagent.
6. Laboratory temperature is important. Please make sure that the kit incubates at a temperature between 22°C and 24°C.

References

1. Blaner, W.S. (1989). Retinol binding protein: the serum transport protein for vitamin A. Endocrine Reviews, 10(3), 308–16.
2. Wolf, G. (1984). Multiple functions of vitamin A. Physiological Reviews, 64(3), 873–937.
3. Petersen, P.A. (1971). Characteristics of a Vitamin A-transporting Protein Complex Occuring in Human Serum. Journal of Biological Chemistry, 246, 34–43.
4. Goodman, D.S. & Blaner, W.S. (1984). In M. B. Sporn, et al. (Eds.). The Retinoids (vol. 2, 1–39). Orlando, FL: Academic Press.
5. Olson, J.A. (1994). Vitamin A, retinoids and carotenoids. In M. E. Shils, et al. (Eds.). Modern Nutrition in Health and Disease (8th ed, 287–307). Philadelphia, PA: Lea & Febiger.

6. Almekinder, J., et al. (2000). Evaluation of plasma retinol-binding protein as a surrogate measure for plasma retinol concentrations. Scandinavian Journal of Clinical and Laboratory Investigation, 60(3), 199–203.
7. Gamble, M.V., et al. (2001). Retinol binding protein as a surrogate measure for serum retinol: Studies in vitamin A-deficient children from the Republic of the Marshall Islands. American Journal of Clinical Nutrition, 73(3), 594–601.
8. Peterson, P.A. & Berggård, I. (1971). Isolation and properties of a human retinol-transporting protein. Journal of Bioligical Chemistry, 246, 25–33.
9. Bernard, A.M. & Lauwerys, R.R. (1981) Retinol-binding protein in urine: A more practical index than urinary β2- microglobulin for the screening of renal tubular function. Clinical Chemistry, 27, 1781–1782.
10. Camara, N.O., et al. (2001). Early detection of heart transplant patients with increased risk of cyclosporin nephrotoxicity. The Lancet, 357, 856-857.
11. Hosaka, B., et al. (2003). Predictive value of urinary retinol-binding protein for graft dysfunction after kidney transplantation. Transplantation Proceedings, 35, 1341–1343.