Quantikine® ELISA

Human RBP4 Immunoassay

Catalog Number DRB400

For the quantitative determination of human Retinol-Binding Protein 4 (RBP4) concentrations in cell culture supernates, serum, plasma, saliva, and urine.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

Retinol-binding protein 4 (RBP4), also known as plasma retinol-binding protein, is a lipocalin superfamily molecule that transports vitamin A (retinol) in the serum (1-4). Dietary retinol is metabolized to retinaldehyde, multiple isomers of retinoic acid, and retinyl esters (1, 5). Retinaldehyde is the critical chromophore in the rhodopsin photoreceptor, while both it and retinoic acid regulate a multitude of cellular differentiation and proliferation effects through the intracellular receptors RAR and RXR (6-8). RBP4 adopts a β-barrel structure with a central cavity that accommodates either retinol or retinaldehyde (9). RBP4 is synthesized primarily by hepatocytes and adipocytes as a 21 kDa non-glycosylated, non-phosphorylated, and non-sulfated molecule (10-12). Its secretion into the blood requires the presence of retinol (10). Proteolytic processing of RBP4 removes one or both C-terminal leucine residues, resulting in 182 and 181 amino acid (aa) forms (12). Human RBP4 shares 100% aa sequence identity with chimpanzee, 91% - 93% aa sequence identity with bovine, porcine, and rabbit, and 83-86% aa sequence identity with chicken, mouse, and rat RBP4, respectively.

The RBP4-retinol complex interacts with transthyretin (TTR), also known as thyroxine-binding protein and prealbumin (2, 13). Formation of this complex increases the serum half-life of RBP4 by preventing RBP4 filtration through the kidney (14). The C-terminally processed forms of RBP4, which do not bind TTR, are normally excreted into the urine but accumulate in the serum during renal failure (12, 13). Glomerular re-uptake of RBP4 is mediated by the endocytic receptor megalin (15). RBP4 is internalized by extrahepatic tissues through a receptor mediated process (16). Vitamin A derivatives in the form of retinyl esters can also be transported in chylomicrons, consistent with the observation that RBP4 deficiency results in only minor clinical effects (5, 14, 17, 18).

RBP4 promotes hyperglycemia through downregulation of the glucose transporter GLUT4 in adipocytes, upregulation of the hepatic gluconeogenic enzyme PEPCK, and attenuation of insulin receptor signaling in skeletal muscle (19, 20). Serum RBP4 levels are elevated in type 2 diabetes and obesity, due primarily to increased production by visceral and liver adipocytes (19, 21, 22). Increases in serum RBP4 mirror changes in several other parameters linked with those diseases (20, 23). Polymorphisms within the RBP4 gene are also associated with increased serum levels and risk of type 2 diabetes (24). The expression and secretion of adipocyte RBP4 is inhibited by TNF-α and atrial natriuretic peptide, while PPARγ agonists have been shown to have both positive and negative effects on RBP4 levels (19, 25, 26).

The Quantikine® Human RBP4 immunoassay is a 2.5 hour solid phase ELISA designed to measure human RBP4 in cell culture supernates, serum, plasma, urine, and saliva. It contains NS0-expressed recombinant human RBP4 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human RBP4 showed linear curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for natural human RBP4.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human RBP4 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any RBP4 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human RBP4 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of RBP4 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human RBP4 Microplate	893266	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human RBP4.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human RBP4 Conjugate	893267	21 mL of a monoclonal antibody specific for human RBP4 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human RBP4 Standard	893268	Recombinant human RBP4 in a buffered protein solution with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-118	896001	2 vials (11 mL/vial) of a buffered protein solution with preservatives.	
Calibrator Diluent RD5-50	895917	21 mL of a concentrated buffered protein base with preservatives and blue dye. *Use diluted 1:5 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Collection device for saliva samples that has no protein binding or filtering capabilities such as Salivette® or equivalent.
- Test tubes for dilution of standards and samples.
- Human RBP4 Controls (optional; R&D Systems®, Catalog # QC23).

PRECAUTIONS

RBP4 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

Saliva - Collect saliva using a collection device such as a Salivette or equivalent. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Saliva collector must not have any protein binding or filtering capabilities.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Cell culture supernate samples may require dilution.

Serum and plasma samples require a 1000-fold dilution. A suggested 1000-fold dilution can be achieved by adding 20 µL of sample to 980 µL of Calibrator Diluent RD5-50 (diluted 1:5)*. Mix well. Complete the 1000-fold dilution by adding 25 µL of the diluted solution to 475 µL of Calibrator Diluent RD5-50 (diluted 1:5)*.

Urine samples require a 5-fold dilution. A suggested 5-fold dilution is 100 µL of sample + 400 µL of Calibrator Diluent RD5-50 (diluted 1:5)*.

Saliva samples require a 2-fold dilution. A suggested 2-fold dilution is 50 µL of sample + 50 µL of Calibrator Diluent RD5-50 (diluted 1:5)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *RBP4 is found in saliva. It is recommended that a face mask be used to protect kit reagents from contamination.*

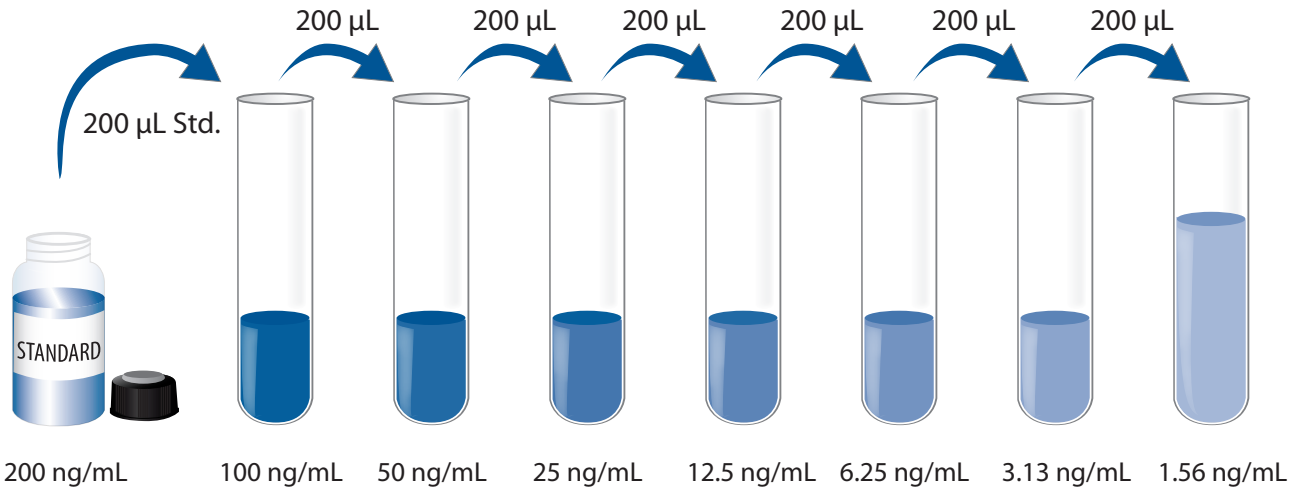
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-50 (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5-50 to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5-50 (diluted 1:5).

Human RBP4 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human RBP4 Standard with deionized or distilled water. This reconstitution produces a stock solution of 200 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD5-50 (diluted 1:5) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 100 ng/mL standard serves as the high standard. Calibrator Diluent RD5-50 (diluted 1:5) serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

Note: *RBP4 is found in saliva. It is recommended that a face mask be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 200 µL of Assay Diluent RD1-118 to each well.
4. Add 20 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) **set at 500 rpm.**

   Note: *In order to obtain the correct signal, it is crucial that the shaker be set to 500 rpm.*
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human RBP4 Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

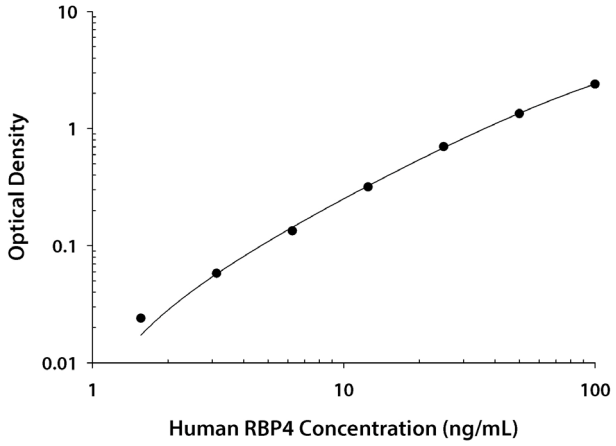
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human RBP4 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D.	Average	Corrected
0	0.006 0.006	0.006	—
1.56	0.029 0.030	0.030	0.024
3.13	0.062 0.065	0.064	0.058
6.25	0.134 0.146	0.140	0.134
12.5	0.320 0.326	0.323	0.317
25	0.700 0.714	0.707	0.701
50	1.306 1.392	1.349	1.343
100	2.385 2.427	2.406	2.400

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (ng/mL)	11.0	30.3	61.9	9.80	27.5	54.7
Standard deviation	0.77	1.73	5.01	0.84	1.60	4.00
CV (%)	7.0	5.7	8.1	8.6	5.8	7.3

RECOVERY

The recovery of human RBP4 spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	102	86-115%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human RBP4 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Saliva (n=4)	Urine (n=4)
1:2	Average % of Expected	103	101	96	103	99	95
	Range (%)	102-104	96-104	88-103	90-108	93-107	87-104
1:4	Average % of Expected	100	96	101	104	103	99
	Range (%)	96-113	91-102	92-106	95-113	99-106	88-106
1:8	Average % of Expected	95	92	95	97	100	98
	Range (%)	93-112	83-102	86-100	87-103	95-105	93-104
1:16	Average % of Expected	95	87	93	95	———	95
	Range (%)	91-107	86-102	89-99	84-108	———	85-112

SENSITIVITY

Fifty assays were evaluated and the minimum detectable dose (MDD) of human RBP4 ranged from 0.053-0.628 ng/mL. The mean MDD was 0.224 ng/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human RBP4 produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human RBP4 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=35)	26,500	12,700-48,600	7700
EDTA plasma (n=35)	25,200	11,900-48,300	7200
Heparin plasma (n=35)	25,000	12,200-43,000	7500
Urine (n=11)	85.2	16.4-252	66.8
Saliva (n=12)	14.9	3.11-29.6	10.0

Cell Culture Supernates:
Human peripheral blood mononuclear cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal bovine serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 1 and 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of human RBP4. No detectable levels were observed.

HepG2 human hepatocellular carcinoma cells (1 x 10^6 cells/mL) were cultured in MEM supplemented with non-essential amino acids, 10% fetal bovine serum, and 2 mM L-glutamine for 5 days. An aliquot of the cell culture supernate was removed, assayed for human RBP4, and measured 75.2 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human RBP4.

The factors listed below were prepared at 1.0 µg/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 1.0 µg/mL in a mid-range recombinant human RBP4 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Lipocalin-1
Lipocalin-2
Prealbumin (transthyretin)
Prealbumin + retinol
Prealbumin + retinol + thyroxine
Retinol

Recombinant mouse:

Lipocalin-2

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