

## Human RBP4 Sandwich ELISA Kit Datasheet

Please read it entirely before use

**Catalogue Number:** KE00270

**Size:** 5\*96T

**Sensitivity:** 0.083 ng/mL

**Range:** 1.56-100 ng/mL

**Usage:** For the quantitative detection of human RBP4 concentrations in serum, plasma, cell culture supernatant, urine and saliva.

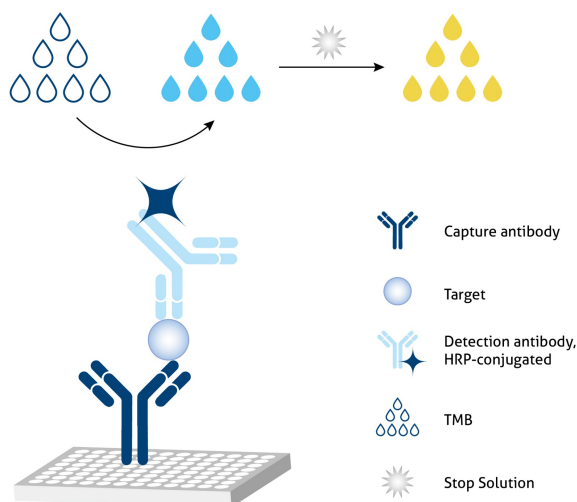
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<b>Table of content</b>	<b>page</b>
1. Background .....	3
2. Principle .....	3
3. Required Materials .....	3
4. Kit Components and Storage .....	4
5. Safety Notes .....	4
6. Sample Collection and Storage .....	4
7. Regent Preparation .....	5
8. Assay Procedure Summary .....	6
9. Validation Data .....	7
9.1 Standard curve .....	7
9.2 Precision .....	8
9.3 Recovery .....	8
9.4 Sample values .....	9
9.5 Sensitivity .....	9
9.6 Linearity .....	9
10. References .....	10

## 1. Background

RBP4 (retinol-binding protein 4) belongs to the lipocalin family and is the specific carrier for retinol (Vitamin A) in the blood. It is mainly synthesized in the liver and circulates in the bloodstream bound to retinol in a complex with transthyretin. RBP4 acts as a drug target for ophthalmology research and reduction in serum RBP4 level inhibits lipofuscin without inhibiting the visual cycle. Urinary RBP4 could act as a marker for glomerular disease. And RBP4 also was identified as an adipokine that elevated in some insulin-resistant states. Measurement of serum RBP4 could be used to assess the risk of insulin resistance, type 2 diabetes, obesity, and cardiovascular disease.

## 2. Principle



### Sandwich ELISA structure (Detection antibody labeled with HRP)

A capture antibody is pre-coated onto the bottom of wells which binds to analyte of interest. A detection antibody labeled with HRP also binds to the analyte. TMB acts as the HRP substrate and the solution color will change from colorless to blue. A stop solution containing sulfuric acid turns solution yellow. The color intensity is proportional to the quantity of bound protein which is measurable at 450 nm with the correction wavelength set at 630 nm.

## 3. Required Materials

- 3.1 A microplate reader capable of measuring absorbance at 450 nm with the correction wavelength set at 630 nm.
- 3.2 Calibrated, adjustable precision pipettes and disposable plastic tips. A manifold multi-channel pipette is recommended for large assays.
- 3.3 Plate washer: automated or manual.
- 3.4 Absorbent paper towels.
- 3.5 Glass or plastic tubes to prepare standard and sample dilutions.
- 3.6 Beakers and graduated cylinders.
- 3.7 Log-log or semi-log graph paper or computer and software for ELISA data analysis. A four-parameter logistic (4-PL) curve-fit is recommended.

## 4. Kit Components and Storage

Microplate - antibody coated 96-well microplate (8 well × 12 strips)	5 plates	<b>Unopened Kit:</b> Store at 2-8°C for 6 months or -20°C for 12 months.  <b>Opened Kit:</b> All reagents stored at 2-8°C for 7 days.  <b>Please use a new standard for each assay.</b>
Protein standard - 200 ng/bottle; lyophilized	10 bottles	
Detection antibody, HRP-conjugated (100×) - 600 μL/vial*	1 vial	
Sample Diluent PT 4 - 150 mL/bottle. For human serum, plasma and urine samples	2 bottles	
Sample Diluent PT 1-ef - 150 mL/bottle. For cell culture supernatant and saliva samples	1 bottle	
Detection Diluent - 150 mL/bottle	1 bottle	
Wash Buffer Concentrate (20×) - 150 mL/bottle	1 bottle	
Tetramethylbenzidine Substrate (TMB) - 60 mL/bottle	1 bottle	
Stop Solution - 60 mL/bottle	1 bottle	
Plate Cover Seals	15 pieces	

\* Centrifugation immediately before use

## 5. Safety Notes

- 5.1 Avoid any skin and eye contact with Stop Solution and TMB. In case of contact, wash thoroughly with water.
- 5.2 Do not use the kit after the expiration date.
- 5.3 Do not mix or substitute reagents or materials from other kit lots or other sources.
- 5.4 Be sure to wear protective equipment such as gloves, masks and goggles during the experiment.
- 5.5 When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer to improve assay precision

## 6. Sample Collection and Storage

- 6.1 Serum: Allow blood samples to clot for 30 minutes, followed by centrifugation for 15 minutes at 1000×g. Clear serum can be assayed immediately or aliquoted and stored at -20°C. Avoid repeated freeze-thaw cycles.
- 6.2 Plasma: Use EDTA, heparin, or citrate as an anticoagulant for plasma collection. Centrifuge for 15 minutes at 1000×g within 30 minutes of collection. The plasma can be assayed immediately or aliquoted and stored at -20°C. Avoid repeated freeze-thaw cycles.
- 6.3 Cell Culture Supernatant: Remove particulates by centrifugation for 5 minutes at 500×g and assay immediately or aliquot and store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.
- 6.4 Urine: Collect urine samples and centrifuge for 20 minutes at 1000×g. Collect the aqueous layer, assay immediately or aliquot and store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.
- 6.5 Saliva: Collect saliva samples and centrifuge for 5 minutes at 10,000×g. Collect the aqueous layer, assay immediately or aliquot and store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

## 7. Regent Preparation

**7.1 Wash Buffer (1X):** If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 30 mL of Wash Buffer Concentrate(20X) to 570 mL deionized or distilled water to prepare 1X Wash Buffer.

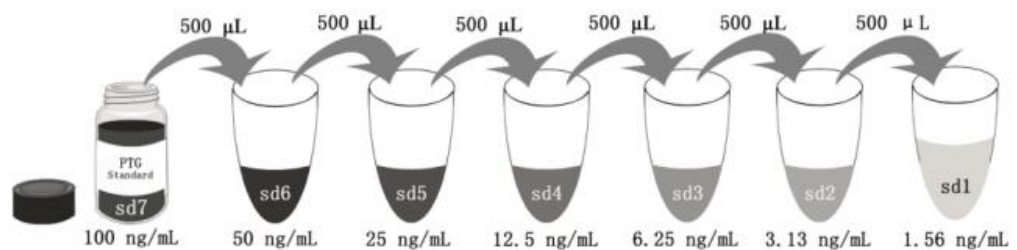
**7.2 Detection Antibody ,HRP-conjugated(1X):** Dilute 100X Detection Antibody, HRP-conjugated 1:100 using Detection Diluent prior to assay. Suggested 1:100 dilution: 10  $\mu$  L 100X Detection Antibody, HRP-conjugated + 990  $\mu$  L Detection Diluent (Centrifuge the 100 X Detection Antibody, HRP-conjugated for a few seconds prior to use).

**7.3 Sample Dilution:** Different samples should be diluted with corresponding Sample Diluent, samples may require further dilution if the readout values are higher than the highest standard OD reading. Variations in sample collection, processing and storage may affect the results of the measurement.

Recommended Dilution for different sample types: 1:2,000 or 1:4,000 is recommended for human serum and plasma; 1:2 to 1: 64 is recommended for cell culture supernatant. 1:4 or 1:8 is recommended for urine, 1:2 or 1:4 is recommended for saliva.

### 7.4 Standard Serial Dilution:

For human serum, plasma and urine samples, add 2 mL Sample Diluent PT 4 in protein standard; For cell culture supernatant and saliva samples add 2 mL Sample Diluent PT 1-ef in protein standard.



Add # $\mu$ L of Standard diluted in the previous step	—	500 $\mu$ L	500 $\mu$ L	500 $\mu$ L	500 $\mu$ L	500 $\mu$ L	500 $\mu$ L
# $\mu$ L of Sample Diluent PT 4 or PT 1-ef	2000 $\mu$ L	500 $\mu$ L	500 $\mu$ L	500 $\mu$ L	500 $\mu$ L	500 $\mu$ L	500 $\mu$ L
	"sd7"	"sd6"	"sd5"	"sd4"	"sd3"	"sd2"	"sd1"

## 8. Assay Procedure Summary

Bring all reagents to room temperature before use (Detection antibody, HRP-conjugated can be used immediately). 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.

8.1 Take out the required number of microplate strips and return excess strips to the foil pouch containing the drying reagent pack and reseal; store at 4°C immediately. Microplate strips should be used in one week.

8.2 Preset the layout of the microplate, including control group, standard group and sample group, add 100  $\mu$  L of each standard and sample to the appropriate wells. (Make sure sample addition is uninterrupted and completed within 5 to 10 minutes, it is recommended to assay all standards, controls, and samples in duplicate).

8.3 Seal plate with cover seal, pressing it firmly onto top of microwells. Incubate the plate for 1 hour at 37°C.

### 8.4 Wash

1) Gently remove the cover seal. Discard the liquid from wells by aspirating or decanting. Remove any residual solution by tapping the plate a few times on fresh paper towels.

2) Wash 4 times with 1X Wash Buffer, using at least 350-400  $\mu$  L per well. Following the last wash, firmly tap plates on fresh towels 10 times to remove residual Wash Buffer. Avoid getting any towel fibers in the wells or wells drying out completely.

8.5 Add 100  $\mu$  L of 1X Detection antibody, HRP-conjugated solution (refer to Reagent Preparation 7.2) to each well. Seal plate with cover seal and incubate for 40 minutes at 37°C.

8.6 Repeat wash step in 8.4.

8.7 Signal development: Add 100  $\mu$  L of TMB substrate solution to each well, protected from light. Incubate for 15 to 20 minutes. Substrate Solution should remain colorless until added to the plate.

8.8 Quenching color development: Add 100  $\mu$  L of Stop Solution to each well in the same order as addition of the TMB substrate. Mix by tapping the side of the plate gently. NB: Avoid skin and eye contact with the Stop solution.

8.9 Read results: Immediately after adding Stop solution read the absorbance on a microplate reader at a wavelength of 450 nm. If possible, perform a double wavelength readout (450 nm and 630 nm).

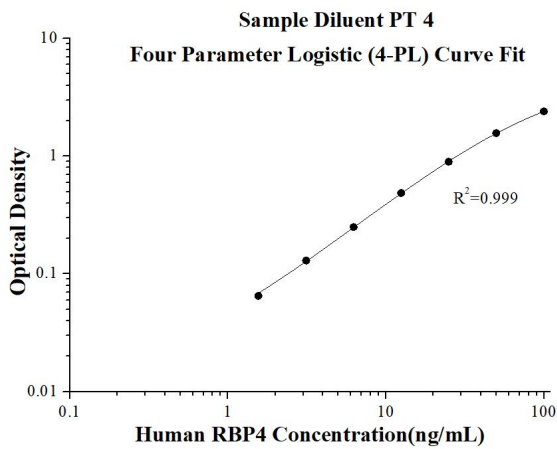
8.10 Data analysis: Calculate the average of the duplicate readings (OD value) for each standard and sample, and subtract the average of the zero standard absorbance. Construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, use four-parameter logistic curve-fit (4-PL) analysis to do this. If the samples have been diluted, the OD readout from the standard curve must be multiplied by the dilution factor used.

Step	Reagent	Volume	Incubation	Wash	Notes
1	Standard and Samples	100 $\mu$ L	60 min	4 times	Cover Wells incubate at 37°C
2	Diluent Detection antibody, HRP-conjugated Solution	100 $\mu$ L	40 min	4 times	Cover Wells incubate at 37°C
3	TMB Substrate	100 $\mu$ L	15-20 min	Do not wash	Incubate in the dark at 37°C
4	Stop Solution	100 $\mu$ L	0 min	Do not wash	-
5	Read plate at 450 nm and 630 nm immediately after adding Stop solution. DO NOT exceed 5 minutes.				

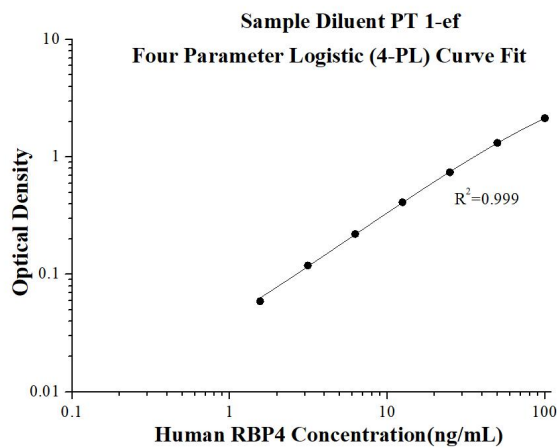
## 9. Validation Data

### 9.1 Standard curve

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D	Average	Corrected
0	0.102 0.105	0.104	-
1.56	0.173 0.164	0.169	0.065
3.13	0.229 0.237	0.233	0.130
6.25	0.352 0.354	0.353	0.250
12.5	0.58 0.599	0.590	0.486
25	0.979 1.02	1.000	0.896
50	1.664 1.677	1.671	1.567
100	2.489 2.507	2.498	2.395



(ng/mL)	O.D	Average	Corrected
0	0.083 0.098	0.091	-
1.56	0.141 0.157	0.149	0.059
3.13	0.208 0.21	0.209	0.119
6.25	0.3 0.323	0.312	0.221
12.5	0.492 0.513	0.503	0.412
25	0.816 0.846	0.831	0.741
50	1.379 1.438	1.409	1.318
100	2.236 2.226	2.231	2.141

## 9.2 Precision

**Intra-assay Precision** (Precision within an assay) Three samples of known concentration were tested 20 times on one plate to assess intra-assay precision.

**Inter-assay Precision** (Precision between assays) Three samples of known concentration were tested in 24 separate assays to assess inter-assay precision.

Intra-assay Precision					Inter-assay Precision				
Sample	n	Mean (ng/mL)	SD	CV%	Sample	n	Mean (ng/mL)	SD	CV%
1	20	12.3	0.65	5.3	1	24	12.6	0.25	2.0
2	20	18.4	0.75	4.0	2	24	18.0	0.60	3.3
3	20	40.8	0.92	2.3	3	24	37.9	1.68	4.4

## 9.3 Recovery

The recovery of human RBP4 spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type		Average% of Expected	Range (%)
Human serum	1:8,000	89	79-94
	1:16,000	86	74-99
Cell culture supernatant	1:2	78	74-80
	1:4	77	72-81
Urine	1:4	91	78-98
	1:8	86	73-104
Saliva	1:2	103	90-119
	1:4	99	86-118



## 9.4 Sample values

Samples from healthy volunteers were evaluated for human RBP4 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean of Detectable (ng/mL)	Range (ng/mL)
Human serum (n=16)	16,353.2	7,552.8-29,470.5
Urine (n=8)	14.9	1.1-29.9
Saliva (n=8)	3.0	0.1-21.8

### Cell culture supernatant

HepG2 were cultured in DMEM supplemented with 10% fetal bovine serum, 2.5 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. An aliquot of the cell culture supernate was removed, assayed for human RBP4, and measured 172.2 ng/mL.

## 9.5 Sensitivity

The minimum detectable dose of human RBP4 is 0.083 ng/mL. This was determined by adding two standard deviations to the concentration corresponding to the mean O.D. of 20 zero standard replicates.

## 9.6 Linearity

To assess the linearity of the assay, samples were diluted with the appropriate **Sample Diluent** to produce samples with values within the dynamic range of the assay. (The human serum was initially diluted 1:1000, cell culture supernatant was initially diluted 1:32)

		Human serum (Sample Diluent PT 4)	Cell culture supernatant (Sample Diluent PT 1-ef)	Urine (Sample Diluent PT 4)	Saliva (Sample Diluent PT 1-ef)
1:2	Average% of Expected	100	100	100	100
	Range (%)	-	-	-	-
1:4	Average% of Expected	115	108	116	117
	Range (%)	106-120	107-109	112-122	110-120
1:8	Average% of Expected	107	101	100	98
	Range (%)	101-114	98-105	74-113	73-111
1:16	Average% of Expected	98			
	Range (%)	82-119			

## 10. References

1. P A Peterson. et al. (1971). J Biol Chem. 246(1):44-9.
2. Yang Q. et al. (2005). Nature. 436(7049):356-62.
3. Frey SK. et al. (2008). Lipids Health Dis. 7(29).
4. Christopher L Cioffi. et al. (2014). J Med Chem. 57(18):7731-57.