

HUMAN RETINOL BINDING PROTEIN 4 (RBP4) ELISA KIT
96-Well Plate (Cat. # EZHRBP4-18K)

I. Intended Use	2
II. Principles Of Procedure	2
III. Reagents Supplied	2
IV. Storage and Stability	4
V. Reagent Precautions	4
VI. Materials Required But Not Provided	4
VII. Sample Collection And Storage	5
VIII. Sample Preparation	5
IX. Standard and Quality Control Preparation	6
X. Assay Procedure	7
XI. Microtiter Plate Arrangement	10
XII. Calculations	11
XIII. Interpretation	11
XIV. Standard Curve	12
XV. Assay Characteristics	13
XVI. Quality Controls	15
XVII. Troubleshooting Guide	16
XVIII. Replacement Reagents	16
XIX. Ordering Information	17

HUMAN RBP4 ELISA KIT
96-Well Plate (Cat. # EZHRBP4-18K)

I. INTENDED USE

This Human Retinol Binding Protein 4 (RBP4) ELISA kit is used for the non-radioactive quantification of Human RBP4 in serum, plasma, and cell culture samples. One kit is sufficient to measure 38 unknown samples in duplicate.

This kit is for research purpose only.

II. PRINCIPLES OF PROCEDURE

This assay is a Sandwich ELISA based, sequentially, on: 1) capture of Human RBP4 molecules from samples to the wells of a microtiter plate coated with anti-human RBP4 antibody, 2) washing of unbound materials from samples, 3) binding of a second biotinylated anti-human RBP4 antibody to the captured molecules, 4) washing of unbound materials from samples, 5) binding of streptavidin-horseradish peroxidase conjugate to the immobilized biotinylated antibodies, 6) washing of excess of free enzyme conjugates, and 7) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbance at 450 nm – 590 nm after acidification of formed products. Since the increase in absorbance is directly proportional to the amount of captured Human RBP4 in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of Human RBP4.

III. REAGENTS SUPPLIED

Each kit is sufficient to run one 96-well plate and contains the following reagents:

A. Human RBP4 ELISA Plate

Coated with Polyclonal anti-Human RBP4 Antibody

Quantity: 1 strip plate

Preparation: Ready to Use

Note: Unused strips should be resealed in the foil pouch with the dessicant provided and stored at 2-8 °C.

B. Adhesive Plate Sealer

Quantity: 2 sheets

Preparation: Ready to Use

III. REAGENTS SUPPLIED (continued)

C. 10X Concentrate HRP Wash Buffer

10X concentrate of 50 mM Tris Buffered Saline containing Tween-20

Quantity: 2 bottles containing 50 mL each

Preparation: Dilute 1:10 with distilled or deionized water

D. Human RBP4 Standard

Purified Recombinant Human RBP4, lyophilized

Quantity: 0.5mL upon hydration

Preparation: Reconstitute with 0.5mL distilled or deionized water.

E. Quality Controls 1 and 2

One vial each, lyophilized, containing recombinant human RBP4 at two different levels.

Quantity: 0.5mL upon hydration

Preparation: Reconstitute each control with 0.5mL distilled or deionized water.

F. Assay Buffer (Sample Diluent)

0.05M Phosphosaline containing 0.025M EDTA, 0.08% Sodium Azide, 1% BSA

Quantity: 2 bottles containing 40 mL each

Preparation: Ready to Use

G. Assay Running Buffer

0.05M Phosphosaline containing 0.025M EDTA, 0.08% Sodium Azide, 1% BSA

Quantity: 10 mL

Preparation: Ready to Use

H. Human RBP4 Detection Antibody

Pre-titered Biotinylated anti-Human RBP4 Antibody

Quantity: 6mL

Preparation: Ready to Use

I. Enzyme Solution

Pre-titered Streptavidin-Horseradish Peroxidase Conjugate in Buffer

Quantity: 12 mL

Preparation: Ready to Use

J. Substrate (Light sensitive, avoid unnecessary exposure to light)

3, 3', 5, 5'-tetramethylbenzidine in buffer

Quantity: 12 mL

Preparation: Ready to Use.

K. Stop Solution (Caution: Corrosive Solution)

0.3 M HCl

Quantity: 12 mL

Preparation: Ready to Use

IV. STORAGE AND STABILITY

Prior to use, all components in the kit can be stored up to 2 weeks at 2-8°C. For longer storage (> 2 weeks), freeze diluted HRP Wash Buffer, Assay Buffer, and reconstituted RBP4 Standards and Controls at $\leq -20^{\circ}\text{C}$. Minimize repeated freeze and thaw of the RBP4 Standards and Quality Controls. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

V. REAGENT PRECAUTIONS

A. Sodium Azide

Sodium Azide has been added to certain reagents as a preservative. Although the concentrations are low, Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

B. Hydrochloric Acid

Hydrochloric Acid is corrosive and can cause eye and skin burns. It is harmful if swallowed and can cause respiratory and digestive tract burns. Avoid contact with skin and eyes. Do not swallow or ingest.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipettes and Pipette Tips: 10 μL - 20 μL or 20 μL - 100 μL
2. Multi-Channel Pipettes and Pipette Tips: 5 ~ 50 μL and 50 ~ 300 μL
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. Deionized Water
6. Microtiter Plate Reader capable of reading absorbency at 450 nm
7. Orbital Microtiter Plate Shaker
8. Absorbent Paper or Cloth

VII. SAMPLE COLLECTION AND STORAGE

1. To prepare serum samples, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. Let blood clot at room temperature for 30 min.
2. Promptly centrifuge the clotted blood at 2,000 to 3,000 x g for 15 minutes at $4 \pm 2^{\circ}\text{C}$.
3. Transfer and store serum samples in separate tubes. Date and identify each sample.
4. Use freshly prepared serum or aliquot and store samples at $\leq -20^{\circ}\text{C}$ for later use. For long-term storage, keep at -70°C . Avoid freeze/thaw cycles.
5. To prepare plasma samples, whole blood should be collected into centrifuge tubes containing enough K_3EDTA to achieve a final concentration of 1.735 mg/mL and centrifuged immediately after collection. Observe the same precautions in the preparation of serum samples.
6. If heparin is to be used as an anticoagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.
7. Avoid using samples with gross hemolysis or lipemia.

VIII. SAMPLE PREPARATION

1. Allow all the reagents to come to room temperature.
2. Dilute serum or plasma samples 1:2000 in Assay Buffer (AB-P). Recommended dilution: dilute 10 μL of serum or plasma in 390 μL of Assay Buffer (AB-P) for a 1:40 dilution. Mix well and dilute 10 μL of dilution 1 into 490 μL of Assay Buffer (AB-P) for a final 1:2000 dilution. Cellular extract and culture media dilutions will vary.

IX. STANDARD AND QUALITY CONTROL PREPARATION

A. Standard Preparation

Human RBP4 Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Human RBP4 Standard with 0.5 mL distilled or deionized water to give a concentration described in the analysis sheet. Invert and mix gently, let sit for 5 minutes then vortex gently.
2. Label six tubes 1, 2, 3, 4, 5, and 6. Add 200 μ L Assay Buffer (AB-P) to each of the six tubes. Perform 3 time serial dilutions by adding 100 μ L of the reconstituted standard to Tube 1, mix well and transfer 100 μ L of Tube 1 to Tube 2, mix well and transfer 100 μ L of Tube 2 to Tube 3, mix well and transfer 100ul of Tube 3 to Tube 4, mix well and transfer 100 μ L of Tube 4 to Tube 5, mix well and transfer 100 μ L of Tube 5 to Tube 6 and mix well.

Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. Unused portions of standard should be stored at $\leq -20^{\circ}\text{C}$. Avoid multiple freeze/thaw cycles.

Volume of Deionized Water to Add	Volume of Standard to Add	Standard Concentration (ng/mL)
0.5 mL	0	X (refer to analysis sheet For exact concentration)

Tube #	Volume of Assay Buffer (AB-P) to Add	Volume of Standard to Add	Standard Concentration (ng/mL)
Tube 1	200 μ L	100ul of reconstituted standard	X/3
Tube 2	200 μ L	100 μ L of Tube 1	X/9
Tube 3	200 μ L	100 μ L of Tube 2	X/27
Tube 4	200 μ L	100 μ L of Tube 3	X/81
Tube 5	200 μ L	100 μ L of Tube 4	X/243
Tube 6	200 μ L	100 μ L of Tube 5	X/729

B. Human RBP4 Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Using a pipette, reconstitute each of the Human RBP4 Quality Control 1 and Quality Control 2 with 0.5 mL distilled or deionized water into the glass vials. Invert and mix gently, let sit for 5 minutes then mix well.

X. ASSAY PROCEDURE

Pre-warm all reagents to room temperature prior to setting up the assay.

1. Dilute the 10X Wash Buffer concentrate 10 fold by mixing the entire content of each bottle of Wash Buffer with 450 mL deionized or distilled water (dilute both bottles with 900 mL deionized water).
2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8°C. Assemble the strips in an empty plate holder and wash each well 3 times with 300 μ L of 1X HRP wash buffer per wash. Decant wash buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. **Do not let wells dry before proceeding to the next step. If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.**
3. Add in duplicate 25 μ L **Assay Running Buffer (EARB)** to all wells.
4. Add in duplicate additional 25 μ L **Assay Running Buffer (EARB)** to blank wells.
5. Add in duplicate 25 μ L Human RBP4 Standards in the order of ascending concentration to the appropriate wells. Add in duplicate 25 μ L QC1 and 25 μ L QC2 to the appropriate wells.
6. Add in sequentially 25 μ L of the diluted unknown samples in duplicate to the remaining wells. **For best result all additions should be completed within 30 minutes.**
7. Cover the plate with plate sealer and incubate at room temperature for 1.5 hours on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 400 to 500 rpm.
8. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells.
9. Wash wells 3 times with 1X Wash Buffer, 300 μ L per well per wash. Decant and tap firmly after each wash to remove residual buffer.
10. Add 50 μ L of Detection Antibody to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 1 hour on the microtiter plate shaker.
11. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells.
12. Wash wells 3 times with 1X Wash Buffer, 300 μ L per well per wash. Decant and tap firmly after each wash to remove residual buffer.

X. ASSAY PROCEDURE (continued)

13. Add 100 μ L Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.
14. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells.
15. Wash wells 3 times with 1X Wash Buffer, 300 μ L per well per wash. Decant and tap firmly after each wash to remove residual buffer.
16. Add 100 μ L of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for 5 to 20 minutes at room temperature. Blue color should be formed in wells of Human RBP4 standards with intensity proportional to increasing concentrations of Human RBP4.

Note: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

17. Remove sealer and add 100 μ L Stop Solution [**CAUTION: CORROSIVE SOLUTION**] and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification. Read absorbance at 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any well. Record the difference of absorbance units. The absorbance of highest RBP4 standard should be approximately 2.2-2.8, or not to exceed the capability of the plate reader used.

Assay Procedure for Human RBP4 ELISA Kit (Cat. # EZHRBP4-18K)

	Step 1	Step 2	Step 3-4	Step 5-6	Step 7-9	Step 10	Step 11-12	Step 13	Step 14-15	Step 16	Step 16	Step 17	Step 17
Well #	Dilute each bottle of 10X Wash Buffer with 450mL Deionized Water.	Wash plate 3X with 300 µl 1X Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels	Assay Running Buffer	Standards/ Controls/ Samples	Seal, Agitate, Incubate 1.5 hours at Room Temperature. Wash 3X with 300 µL Wash Buffer	Detection Antibody	Seal, Agitate, Incubate 1 hour at Room Temperature. Wash 3X with 300 µL Wash Buffer	Enzyme Solution	Seal, Agitate, Incubate 30 minutes at Room Temperature. Wash 3X with 300 µL Wash Buffer	Substrate	Seal, Agitate, Incubate 5 - 20 minutes at Room Temperature.	Stop Solution	Read Absorbance at 450 nm and 590 nm.
A1, B1			50 µL			50 µL		100 µL		100 µL			
C1, D1			25 µL	25 µL of Tube 6		↓		↓		↓			
E1, F1			25 µL	25 µL of Tube 5									
G1, H1			25 µL	25 µL of Tube 4									
A2, B2			25 µL	25 µL of Tube 3									
C2, D2			25 µL	25 µL of Tube 2									
E2, F2			25 µL	25 µL of Tube 1									
G2, H2			25 µL	25 µL of reconstituted standard									
A3, B3			25 µL	25 µL of QC 1									
C3, D3			25 µL	25 µL of QC 2									
E3, F3			25 µL	25 µL of diluted Sample									
G3, H3			25 µL	25 µL of diluted Sample									
A4, B4 ↓	25 µL	25 µL of diluted Sample											

XI. MICROTITER PLATE ARRANGEMENT

Human RBP4 ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Tube 3	QC 1	Etc.								
B	Blank	Tube 3	QC 1	Etc.								
C	Tube 6	Tube 2	QC2									
D	Tube 6	Tube 2	QC2									
E	Tube 5	Tube 1	Sample									
F	Tube 5	Tube 1	Sample									
G	Tube 4	Reconstituted Standard	Sample									
H	Tube4	Reconstituted Standard	Sample									

XII. CALCULATIONS

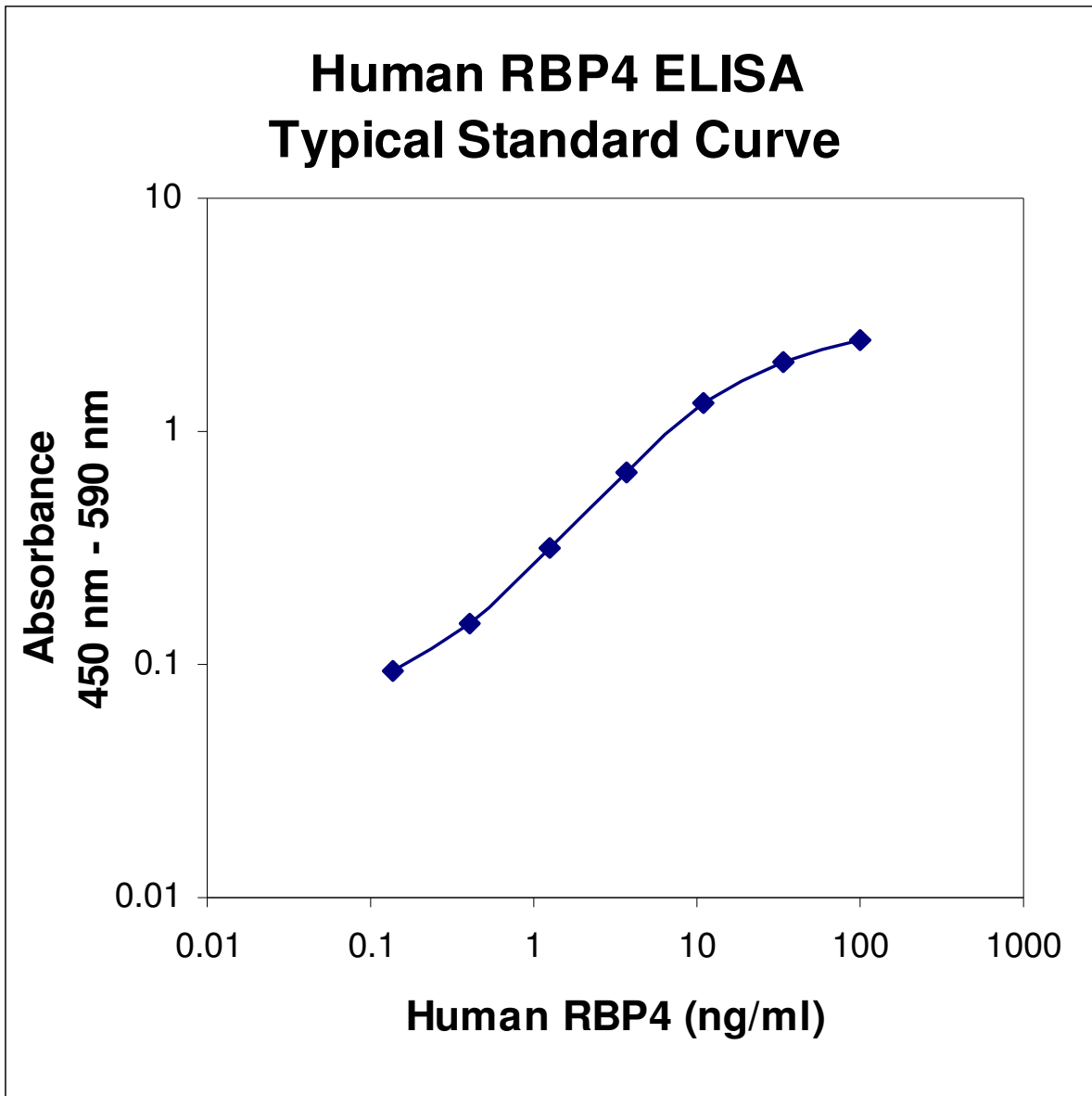
The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function.

Note: When sample volumes assayed differ from 25 μL , an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 50 μL of sample is used, then calculated data must be divided by 2). When sample volume assayed is less than 25 μL , compensate the volume deficit with assay buffer (AB-P).

XIII. INTERPRETATION

1. The assay will be considered accepted when all Quality Control values fall within the calculated Quality Control Range. If any QC's fall outside the control range, review results with a supervisor.
2. If the difference between duplicate results of a sample is $>15\%$ CV, repeat the sample.
3. The limit of sensitivity of this assay is 0.14 ng/mL Human RBP4 (25 μL sample size).
4. The appropriate range of this assay is 0.14 ng/mL to 100 ng/mL Human RBP4 (25 μL sample size). Any result greater than 100 ng/mL in a 25 μL sample should be diluted using assay buffer (AB-P), and the assay repeated until the results fall within range.

XIV. STANDARD CURVE



Typical Standard Curve, not to be used to calculate data.

XV. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Human RBP4 that can be detected by this assay is 0.14 ng/mL when using a 25 μ L sample size.

B. Specificity

The antibody pair used in this assay is specific to Human RBP4. No species cross-reactivity is observed in the assay to mouse, guinea pig, hamster, or rabbit samples. However, there is a high cross-reactivity to non-human primate, canine, feline, and horse samples. This species cross reactivity has not been evaluated or calibrated.

No cross reactivity is observed to the following human analytes: Total Adiponectin, Resistin, PAI-1 (Total), and PAI-1 (Active).

C. Precision

Intra-Assay Variation

Sample No.	Mean RBP4 Levels (ng/mL)	Intra-Assay% CV
1	6.44	7.75
2	8.93	4.30
3	9.77	3.77
4	10.65	6.46
5	11.7	7.41

Inter-Assay Variation

Sample No.	Mean RBP4 Levels (ng/mL)	Inter-Assay % CV
1	8.45	8.06
2	8.78	5.87
3	9.58	3.34
4	10.99	5.27
5	11.28	5.90

The assay variations of Human RBP4 ELISA kits were studied on five human serum samples with varying concentrations of endogenous RBP4. The mean Intra-Assay variation was calculated from results of 8 replicate determinations in each assay of the indicated samples. The mean Inter-Assay variations of each sample were calculated from results of three separate assays with duplicate samples in each assay.

XV. ASSAY CHARACTERISTICS (continued)

D. Recovery

Spike & Recovery of Human RBP4 in Serum

Sample No.	RBP4 Added ng/mL	Expected ng/mL	Observed ng/mL	% of Recovery
1	0	5.60	5.06	
	1.56	7.16	8.10	113.13
	6.25	11.85	12.90	108.86
	25.00	30.60	32.70	106.86
2	0	7.10	7.10	
	1.56	8.66	8.50	98.15
	6.25	13.35	12.80	95.88
	25.00	32.10	31.70	98.75
3	0	7.70	7.70	
	1.56	9.26	8.70	93.95
	6.25	13.95	13.10	93.91
	25.00	32.70	26.20	80.12
4	0	8.50	8.50	
	1.56	10.06	10.00	99.40
	6.25	14.75	13.90	94.24
	25.00	33.50	32.00	95.52
5	0	9.30	9.30	
	1.56	10.86	10.50	96.69
	6.25	15.55	14.80	95.18
	25.00	34.30	33.10	96.50
6	0	10.2	10.20	
	1.56	11.76	11.90	101.19
	6.25	16.45	15.50	94.22
	25.00	35.20	26.90	76.42

Varying amounts of Human RBP4 were added to six human serum samples and the RBP4 content was determined in two separate assays. The % of recovery = observed RBP4 concentrations/expected RBP4 concentrations x 100%.

XV. ASSAY CHARACTERISTICS (continued)

E. Linearity

Effect of Serum Dilution

Sample No.	Volume Sampled	Expected ng/mL	Observed ng/mL	% Of Expected
1	25µL	5.70	5.70	100
	12.5µL	2.85	3.30	115.79
	6.25µL	1.423	1.60	112.28
2	25µL	6.30	6.30	100
	12.5µL	3.15	3.20	101.59
	6.25µL	1.58		
3	25µL	6.50	6.50	100
	12.5µL	3.25	4.10	126.15
	6.25µL	1.63	2.20	135.38
4	25µL	7.80	7.8	100
	12.5µL	3.90	5.2	133.33
	6.25µL	1.96	2.5	128.21
5	25µL	8.90	8.9	100
	12.5µL	4.45	5.2	116.85
	6.25µL	2.23	2.7	121.35
6	25µL	9.4	9.4	100
	12.5µL	4.70	5.1	108.51
	6.25µL	2.35	2.6	110.64

Six human serum samples with the indicated sample volumes were assayed in two separate experiments. Required amounts of assay buffer were added to compensate for lost volumes below 25 µL. The resulting dilution factors of 1, 2, and 4 representing 25 µL, 12.5 µL, and 6.25 µL sample volumes assayed, respectively, were applied in the calculation of % expected = observed/expected x 100%.

XVI. QUALITY CONTROLS

The ranges for Quality Controls 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com.

XVII. TROUBLESHOOTING GUIDE

1. To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.
2. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
3. Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started all steps should be completed with precise timing and without interruption.
4. Avoid cross contamination of any reagents or samples to be used in the assay.
5. Make sure all reagents and samples are added to the bottom of each well.
6. Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or cross well contamination due to inappropriate mixing.
7. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
8. Do not let the absorbency reading of the highest standard reach 3.0 units or higher after acidification.
9. High absorbance in background or blank wells could be due to 1) cross well contamination by standard solution or sample or 2) inadequate washing of wells with HRP Wash Buffer or 3) overexposure to light after substrate has been added.

XVIII. REPLACEMENT REAGENTS

Reagents	Cat. #
Microtiter Plate	EP18
10X HRP Wash Buffer Concentrate (50 mL)	EWB-HRP
Human RBP4 Standard	E8018-K
Quality Controls 1 and 2	E6018-K
Assay Buffer (Sample Diluent)	AB-P
Assay Running Buffer	EARB
Human RBP4 Detection Antibody	E1018
Enzyme Solution	EHRP-4
Substrate	ESS-TMB2
Stop Solution	ET-TMB

XIX. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size

TELEPHONE ORDERS:

Toll Free US (866) 441-8400
(636) 441-8400

FAX ORDERS: (636) 441-8050

MAIL ORDERS: Millipore

6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

To best serve our international customers, it is Millipore's policy to sell our products through a network of distributors. To place an order or to obtain additional information about Millipore products, please contact your local distributor.

B. Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to human or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material safety data sheets for Millipore products may be ordered by fax or phone. See Section A above for details on ordering.