Human Retinol Binding Protein (RBP) Serum EIA Kit

**CATALOG NO:** IRAAKT2535

**LOT NO:** SAMPLE

**INTENDED USE**
The Retinol Binding Protein (RBP) kit is designed to quantitatively measure RBP present in serum and plasma samples.
BACKGROUND

Retinol binding protein (RBP) is from a family of structurally related proteins that bind small hydrophobic molecules such as bile pigments, steroids, odorants, etc.1 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). The structure of retinol is shown below.

![Structure of Retinol](image)

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. 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 reduced^3^4.

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 serum^1,5^6, 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 VAD^7 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. Therefore, individuals with diseases such as cystic fibrosis^8^ and HIV-19 also run the risk of VAD due to the infectious stresses that contribute to the disease.

ASSAY PRINCIPLE
The Retinol Binding Protein (RBP) kit is designed to quantitatively measure RBP present in serum and plasma samples. Please read the complete kit insert before performing this assay. 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 rabbit 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 450nm wavelength. The concentration of the RBP in the sample is calculated, after making a suitable correction for the dilution of the sample, using software available with most plate readers.

SUPPLIED COMPONENTS

- **Coated Clear 96 Well Plate One Plate**: A clear plastic microplate with break-apart strips coated with goat anti-rabbit IgG.
- **RBP Standard 60 μL**: A stock solution of native human RBP at 100 μg/mL.
- **RBP Antibody 3 mL**: A polyclonal antibody specific for RBP.
- **RBP-Peroxidase Conjugate 5 mL**: A RBP-peroxidase conjugate.
- **Assay Buffer 28 mL**
- **Wash Buffer Concentrate 30 mL**: A 20X concentrate that should be diluted with deionized or distilled water.
- **TMB Substrate 11 mL**
- **Stop Solution 11 mL**: A 1N hydrochloric acid solution. Caustic.
- **Plate Sealer 1 each**

STORAGE INSTRUCTIONS

- All components of this kit should be stored at 4°C until the expiration date of the kit.
OTHER MATERIALS REQUIRED
• Distilled or deionized water.
• Repeater pipet with disposable tips capable of dispensing 25 μL, 50 μL and 100 μL.
• A microplate shaker and washer.
• Colorimetric 96 well microplate reader capable of reading optical density at 450 nm.
• Software for converting raw relative optical density readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting.

PRECAUTIONS
• 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.
• 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.
• The RBP Standard is purified from a human source and as such, should be treated as potentially hazardous. Proper safety procedures must be followed.
• 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.
• The Stop Solution is acid. The solution should not come in contact with skin or eyes. Take appropriate precautions when handling this reagent.

SAMPLE TYPES
• This assay has been validated for human serum and EDTA and heparin plasma samples only. Samples containing visible particulate should be centrifuged prior to using. Moderate to severely hemolyzed samples should not be used in this kit. They have been shown to yield artificially high RBP concentrations.
• RBP is a highly conserved protein and we have shown that the Urinary RBP kit, which uses identical antibody and conjugate, will measure RBP’s from sources other than human including rat, dog and rhesus monkey. The end user should evaluate recoveries of RBP in other plasma and serum samples being tested.
SAMPLE PREPARATION

- Samples must be diluted 1:40 by taking one part of serum and adding thirty-nine parts of Assay Buffer prior to running in the kit. Any samples with RBP concentrations outside the standard curve range should be diluted further with Assay Buffer to obtain readings within the standard curve.

Use all samples within 2 hours of dilution.

REAGENT PREPARATION

Allow the kit reagents to come to room temperature for 30 minutes. The recommended format is 1 hr at room temperature with shaking. The assay is sensitive to temperature. Significant changes to temperature during incubation will cause results to vary. We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine RBP concentrations. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

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 for 3 months at room temperature.

Standard Preparation

Label seven tubes #1 through #7. Briefly spin vial of standard in a microcentrifuge to ensure contents are at bottom of vial. Pipet 475 μL of Assay Buffer into tube #1 and 250 μL into tubes #2-#7. Carefully add 25 μL of the RBP Standard stock solution to tube #1 and vortex completely. Take 250 μL of the RBP solution in tube #1 and add it to tube #2 and vortex completely. Add 250 μL of tube #2 to tube #3 and vortex completely. Repeat these serial dilutions for tubes #4 through #7. The concentration of RBP in tubes 1 through 7 will be 5, 2.5, 1.25, 0.625, 0.313, 0.156 and 0.078 μg/mL.
ASSAY PROTOCOL

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 foil pouch with desiccant. Seal the ziploc plate bag and store at 4°C.

2. Pipet 25 μL of samples or standards into wells in the plate. Pipet 50 μL of Assay Buffer into the non-specific binding (NSB) wells. Pipet 25 μL of Assay Buffer into wells to act as maximum binding wells (B0).

3. Add 50 μL of the RBP-peroxidase conjugate to each well, using a repeater pipet.

4. Add 25 μL of the RBP Antibody solution to each well, except the NSB wells, using a repeater pipet.

5. 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.

6. Aspirate the plate and wash each well 4 times with 300 μL wash buffer. Tap the plate dry on clean absorbent towels.

7. Add 100 μL of the TMB Substrate to each well, using a repeater pipet.

8. Incubate the plate at room temperature for 15 minutes.

9. Add 100 μL of the Stop Solution to each well, using a repeater pipet.

10. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.

11. Use the plate reader’s built-in 4PLC software capabilities to calculate RBP concentration for each sample.
CALCULATION OF RESULTS

Average the duplicate OD readings for each standard and sample. Create a standard curve by reducing the data using computer software capable of generating a four-parameter logistic curve (4PLC) fit, after subtracting the mean OD’s for the blank. The sample concentrations, calculated off the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values.

**Typical Data**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean OD</th>
<th>Net OD</th>
<th>% B/B0</th>
<th>RBP Conc. (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSB</td>
<td>0.145</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard 1</td>
<td>0.538</td>
<td>0.393</td>
<td>12.0</td>
<td>5</td>
</tr>
<tr>
<td>Standard 2</td>
<td>0.816</td>
<td>0.671</td>
<td>20.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Standard 3</td>
<td>1.074</td>
<td>0.929</td>
<td>28.5</td>
<td>1.25</td>
</tr>
<tr>
<td>Standard 4</td>
<td>1.594</td>
<td>1.449</td>
<td>44.4</td>
<td>0.625</td>
</tr>
<tr>
<td>Standard 5</td>
<td>2.025</td>
<td>1.881</td>
<td>57.6</td>
<td>0.313</td>
</tr>
<tr>
<td>Standard 6</td>
<td>2.496</td>
<td>2.351</td>
<td>72.0</td>
<td>0.156</td>
</tr>
<tr>
<td>Standard 7</td>
<td>2.830</td>
<td>2.686</td>
<td>82.3</td>
<td>0.078</td>
</tr>
<tr>
<td>BO</td>
<td>3.409</td>
<td>3.264</td>
<td>100.0</td>
<td>0</td>
</tr>
<tr>
<td>Sample 1</td>
<td>1.666</td>
<td>1.521</td>
<td>46.6</td>
<td>0.538</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1.066</td>
<td>0.921</td>
<td>28.2</td>
<td>1.376</td>
</tr>
</tbody>
</table>

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

Conversion Factor: 1 µg/mL of human RBP is equivalent to 47.62 nM RBP
VALIDATION DATA

Sensitivity and Limit of Detection

- Sensitivity was calculated by comparing the OD’s for nineteen wells run for each of the B0 and standard #7. The detection limit was determined at two (2) standard deviations from the B0 along the standard curve.
- Sensitivity was determined as 0.029 μg/mL.

LINEARITY

Linearity was determined by taking two urine samples diluted with Assay Buffer, one with a low diluted progesterone level of 404.1 pg/mL and one with a higher diluted level of 1,774.5 pg/mL, and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

<table>
<thead>
<tr>
<th>Low Serum</th>
<th>High Serum</th>
<th>Observed Conc. (μg/mL)</th>
<th>Expected Conc. (μg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>0%</td>
<td>0.490</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>80%</td>
<td>20%</td>
<td>0.692</td>
<td>0.685</td>
<td>99.0</td>
</tr>
<tr>
<td>60%</td>
<td>40%</td>
<td>0.895</td>
<td>0.905</td>
<td>101.1</td>
</tr>
<tr>
<td>40%</td>
<td>60%</td>
<td>1.097</td>
<td>1.086</td>
<td>99.0</td>
</tr>
<tr>
<td>20%</td>
<td>80%</td>
<td>1.300</td>
<td>1.292</td>
<td>99.4</td>
</tr>
<tr>
<td>0%</td>
<td>100%</td>
<td>1.502</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Mean Recovery 99.6%
Intra Assay Precision

Four human serum samples were diluted 1:40 with Assay Buffer and run in replicates of 19 in an assay. The mean and precision of the calculated RBP concentrations were:

<table>
<thead>
<tr>
<th>Sample</th>
<th>RBP Conc. (μg/mL)</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.48</td>
<td>5.9</td>
</tr>
<tr>
<td>2</td>
<td>1.04</td>
<td>4.2</td>
</tr>
<tr>
<td>3</td>
<td>1.54</td>
<td>4.6</td>
</tr>
<tr>
<td>4</td>
<td>0.77</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Inter Assay Precision

Four human serum samples were diluted 1:40 with Assay Buffer and run in duplicates in twenty-one assays run over multiple days by two operators. The mean and precision of the calculated RBP concentrations were:

<table>
<thead>
<tr>
<th>Sample</th>
<th>RBP Conc. (μg/mL)</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.52</td>
<td>11.2</td>
</tr>
<tr>
<td>2</td>
<td>1.06</td>
<td>8.2</td>
</tr>
<tr>
<td>3</td>
<td>1.55</td>
<td>8.8</td>
</tr>
<tr>
<td>4</td>
<td>0.79</td>
<td>8.5</td>
</tr>
</tbody>
</table>

**SAMPLE VALUES**

Sixteen random human serum and plasma samples were tested in the assay. Values ranged from 23.2 to 35.2 μg/mL with an average of 29.6 μg/mL. The normal reference range for serum RBP is 30-60 μg/mL\(^{10}\). Samples with a serum RBP level below about 15 μg/mL RBP maybe considered Vitamin A deficient\(^{11}\).
CROSS REACTIVITY AND INTERFERENTS

Two serum samples were spiked with varying concentrations of bilirubin, diluted 1:50 in Assay Buffer and tested in the assay. Bilirubin levels in normal serum are between 0.2 and 1.0 mg/dL\textsuperscript{12}. No significant change to the measured RBP levels were observed up to an additional 10.0 mg/dL of bilirubin.

A serum sample was spiked with varying concentrations of hemoglobin, diluted 1:50 in Assay Buffer and tested in the assay. No significant change to the measured RBP level was observed up to an additional 2 mg/mL of hemoglobin. However, moderately to severely hemolyzed samples should be avoided as they have been shown to yield artificially high RBP concentrations.

A serum sample was spiked with varying concentrations of lipids, diluted 1:40 in Assay Buffer and tested in the assay. No significant change to the measured RBP level was observed with the addition of high, medium and low levels of lipids.


