Human Retinol Binding Protein (RBP) Urinary EIA Kit

CATALOG NO: IRAAKT2536
LOT NO: SAMPLE

INTENDED USE
The Urinary Retinol Binding Protein (RBP) kit is designed to quantitatively measure RBP present in urine 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. 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). 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.

RBP has also been shown to be a useful marker for renal function as it is totally filtered by the glomeruli and reabsorbed by proximal tubules. This has made urinary RBP (uRBP) a tool to study renal function in heart or kidney transplant recipients, type 1 and 2 diabetics, and in people exposed to uranium from mining operations. Measurement of uRBP levels has also been useful in detection and characterization of diseases including hypertension and certain cancers.

ASSAY PRINCIPLE
The Urinary Retinol Binding Protein (RBP) kit is designed to quantitatively measure RBP present in urine 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 450 nm 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 20 μg/mL.
- **RBP Antibody 3 mL:** A polyclonal antibody specific for RBP.
- **RBP-Peroxidase Conjugate 3 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**

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.
- A microplate shaker and a microplate washer.
- Colorimetric 96 well microplate reader capable of reading optical density at 450 nm, preferably with correction between 570 and 590 nm.
- 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.

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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 fully validated for human urine samples and tested in rat, dog and rhesus monkey urines. Samples containing visible particulate should be centrifuged prior to using.
- RBP is a highly conserved protein and we have shown that this kit may measure RBP’s from sources other than human. Please see page 14 for details of other urine samples tested. The end user should evaluate recoveries of RBP in other urine samples being tested.

SAMPLE PREPARATION

- Samples must be diluted 1:2 by adding one part of urine to one part Assay Buffer prior to running in the kit. Any samples with RBP concentrations greater than the standard curve range should be diluted further with Assay Buffer to obtain readings within the standard curve. Samples that are too dilute to be measured should be concentrated prior to measuring in the assay.

Use all samples within 2 hours of dilution.
REAGENT PREPARATION
Allow the kit reagents to come to room temperature for 30 minutes. 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 Preparation
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 five tubes #1 through #5. 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 300 μL into tubes #2 to #5. Carefully add 25 μL of the RBP stock solution to tube #1 and vortex completely. Take 100 μL of the RBP solution in tube #1 and add it to tube #2 and vortex completely. Repeat these serial dilutions for tubes #3 through #5. The concentration of RBP in tubes 1 through 5 will be 1,000, 250, 62.5, 15.625, and 3.906 ng/mL.
ASSAY PROTOCOL

1. Use the plate layout sheet on the back page of the kit insert 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 50 μL of samples or standards into wells in the plate. Pipet 75 μL of Assay Buffer into the non-specific binding (NSB) wells. Pipet 50 μL of Assay Buffer into wells to act as maximum binding wells (Bo).

3. Add 25 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 30 minutes without shaking.

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 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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean OD</th>
<th>Net OD</th>
<th>% B/B0</th>
<th>RBP Conc. (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSB</td>
<td>0.061</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard 1</td>
<td>0.171</td>
<td>0.110</td>
<td>9.2</td>
<td>1.000</td>
</tr>
<tr>
<td>Standard 2</td>
<td>0.231</td>
<td>0.270</td>
<td>22.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Standard 3</td>
<td>0.567</td>
<td>0.506</td>
<td>42.5</td>
<td>62.5</td>
</tr>
<tr>
<td>Standard 4</td>
<td>0.875</td>
<td>0.815</td>
<td>68.5</td>
<td>15.625</td>
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<tr>
<td>Standard 5</td>
<td>1.096</td>
<td>1.035</td>
<td>87.0</td>
<td>3.906</td>
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<tr>
<td>R0</td>
<td>1.251</td>
<td>1.190</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Sample 1</td>
<td>0.289</td>
<td>0.228</td>
<td>19.1</td>
<td>32.6</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.729</td>
<td>0.679</td>
<td>57.0</td>
<td>29.27</td>
</tr>
</tbody>
</table>

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

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

Typical Standard Curve

Always run your own standard curves for calculation of results. Do not use this data.
VALIDATION DATA

Sensitivity and Limit of Detection

- Sensitivity was calculated by comparing the OD’s for twenty wells run for each of the B0 and standard #5. The detection limit was determined at two (2) standard deviations from the B0 along the standard curve.
- **Sensitivity was determined as 2.90 ng/mL.**
- The Limit of Detection for the assay was determined in a similar manner by comparing the OD’s for twenty replicates for each of the zero standard and a low concentration human urine sample.
- **Limit of Detection was determined as 4.09 ng/mL*.**
- Note: Due to the dilute nature of this sample it was run neat instead of being diluted 1:2.

LINEARITY

Linearity was determined by taking two human urine samples diluted 1:2, one with a low diluted RBP level of 14.5 ng/mL and one with a higher diluted level of 299.2 ng/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>High Urine</th>
<th>Low Urine</th>
<th>Expected Conc. (ng/mL)</th>
<th>Observed Conc. (ng/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>0%</td>
<td>299.2</td>
<td>299.2</td>
<td>100%</td>
</tr>
<tr>
<td>80%</td>
<td>20%</td>
<td>227.9</td>
<td>227.9</td>
<td>94.1</td>
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<tr>
<td>60%</td>
<td>40%</td>
<td>180.4</td>
<td>180.4</td>
<td>97.3</td>
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<tr>
<td>40%</td>
<td>60%</td>
<td>120.5</td>
<td>120.5</td>
<td>93.9</td>
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<tr>
<td>20%</td>
<td>80%</td>
<td>70.0</td>
<td>70.0</td>
<td>98.0</td>
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<tr>
<td>0%</td>
<td>100%</td>
<td>14.5</td>
<td>14.5</td>
<td>100%</td>
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</tbody>
</table>

Mean Recovery: 95.8%

*Note: Due to the dilute nature of this sample it was run neat instead of being diluted 1:2.
**Intra Assay Precision**
Four human urine samples were diluted 1:2 with Assay Buffer and run in replicates of 8 in an assay. The mean and precision of the calculated RBP concentrations were:

<table>
<thead>
<tr>
<th>Sample</th>
<th>RBP Conc. (ng/mL)</th>
<th>%CV</th>
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<tbody>
<tr>
<td>1</td>
<td>14.8</td>
<td>4.5</td>
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<tr>
<td>2</td>
<td>28.0</td>
<td>7.5</td>
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<td>3</td>
<td>53.5</td>
<td>7.3</td>
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<tr>
<td>4</td>
<td>323.7</td>
<td>2.1</td>
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</table>

**Inter Assay Precision**
Four human urine samples were diluted 1:2 with Assay Buffer and run in duplicates in twenty-one assays run over multiple days by three operators. The mean and precision of the calculated RBP concentrations were:

<table>
<thead>
<tr>
<th>Sample</th>
<th>RBP Conc. (ng/mL)</th>
<th>%CV</th>
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<tbody>
<tr>
<td>1</td>
<td>14.4</td>
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<tr>
<td>2</td>
<td>28.0</td>
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<tr>
<td>3</td>
<td>50.8</td>
<td>8.1</td>
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<td>4</td>
<td>309.2</td>
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**SAMPLE VALUES**

Fourteen random human urine samples were tested in the assay. Values ranged from 6.8 to 788.5 ng/mL with a mean of 114.7 ng/mL. These samples were also run and the RBP levels normalized to creatinine levels. Normalized values ranged from 35.9 to 573.9 μg RBP/g creatinine.

Normal ranges for urinary RBP are < 130 μg RBP/g creatinine for individuals under 50 years of age and < 172 μg RBP/g creatinine for those equal to or older than 5015.

**Other Species**

We have tested a range of urines from other species for RBP. These include rat, dog and rhesus monkey urine. Because of the difficulty in obtaining urine from animals with known medical history, the values we obtained may not be representative of normal or diseased states. Rat urine diluted 1:2 with Assay Buffer had a neat sample value of 43.37 ng/mL and when normalized to urinary creatine gave a reading of 176.1 μg/g creatinine.

Samples of urine from healthy dog and rhesus monkey read below the 3.906 ng/mL standard. We therefore concentrated these samples by freeze drying them and reconstituting them in one-tenth their original volume with Assay Buffer. At that concentration, neat dog urine read at 29.95 ng/mL and when normalized to urinary creatine gave a reading of 32.27 μg/g creatinine. The neat monkey urine read at 10.50 ng/mL and when normalized to urinary creatine gave a reading of 395.5 μg/g creatinine.

# Assay Template

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