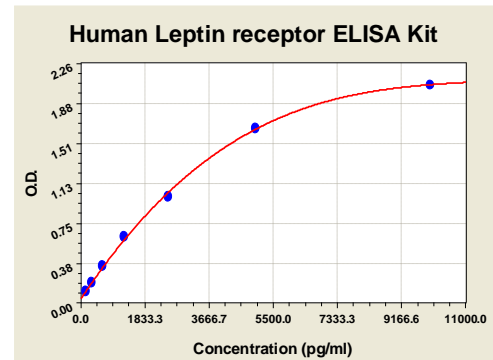


## KIT COMPONENTS

| Component   | Amount               |
|---|----------------------|
| 96-well plate pre-coated with Anti-Human LeptinR        | 1 Plate              |
| Protein Standard: Lyophilized recombinant human LeptinR | 2 tubes, 100 ng/tube |
| Sample Diluent Buffer                                   | 30 ml                |
| Biotinylated Antibody (Anti-Human LeptinR)              | 130 µl (100x)        |
| Antibody Diluent Buffer                                 | 12ml                 |
| Avidin-Biotin-Peroxidase Complex (ABC) Solution         | 130 µl (100x)        |
| ABC Diluent Buffer                                      | 12 ml                |
| Tetramethylbenzidine (TMB) Color Developing Agent       | 10 ml                |
| TMB Stop Solution                                       | 10 ml                |

### Storage

Store at 4°C. Cell Applications, Inc. recommends using the kit within 6 months of order.



| X | pg/ml | 0.0   | 156   | 313   | 625   | 1250  | 2500  | 5000  | 10000 |       |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Y | O.D.  | 0.450 | 0.015 | 0.117 | 0.198 | 0.353 | 0.628 | 1.004 | 1.646 | 2.053 |

**Figure 1: LeptinR ELISA Kit Standard Curve.** Using the Human LeptinR ELISA Kit, O.D. data was graphed against LeptinR protein concentration. The TMB reaction was incubated at 37° C for 8 min.

## LEPTIN RECEPTOR BACKGROUND

Leptin, the 16-kDa protein product of the ob gene, is an adipocyte-derived hormone that influences energy homeostasis through effects on energy intake and expenditure, the secretion and action of metabolic hormones, and neuroendocrine function. Leptin acts through receptors that bear strong sequence homology to the class I cytokine receptor family. Mutations in the leptin receptor gene occur in db/db mice, and fa/fa and Koletsky rats, producing severe obesity with resistance to endogenous and exogenous leptin. At least five isoforms of the leptin receptor are generated by alternative mRNA splicing, but signaling is thought to occur through the "long isoform" (OBRI). OBRI is predicted to have a long intracellular domain of 302 amino acid residues. Three short forms (OBRs) have predicted cytoplasmic domains ranging from 32 to 40 amino acids. These four isoforms have identical extracellular and transmembrane domains. Furthermore, a soluble receptor with no transmembrane region has also been predicted. OBRs is highly expressed in kidney, lungs, and choroid plexus, while OBRI is most abundant in the hypothalamus, but may be expressed at lower levels elsewhere.<sup>1</sup> Leptin binding to the extracellular domain of the LRb dimer activates the Jak2 tyrosine kinase that associates with OBRI via the Box 1 motif and intracellular amino acids 31–36 of LRb. Activated Jak2 tyrosine phosphorylates itself and Tyr985 and Tyr1138 on the intracellular tail of OBRI. Phosphorylated Tyr1138 binds and mediates the phosphorylation-dependent activation of STAT3, which activates SOCS3 (a feedback inhibitor of OBRI signaling) and pro-opiomelanocortin (POMC) (an anorexigenic neuropeptide) and inhibits the transcription of agouti-related protein (AgRP) (an orexigenic neuropeptide). It has been shown that that both OBRs and OBRI had the ability to induce leptin-dependent activation of MAPK in both transiently transfected cells.<sup>2</sup> It is clear that OBRI more robustly activate these pathways than does OBRs. In contrast, only the long form of the leptin receptor had the ability to activate STAT3 tyrosine phosphorylation and to stimulated c-fos gene transcription.<sup>3</sup>

### Reference

- Münzberg, H. et al: Cell. Mol. Life. Sci. 62: 642-52, 2005
- Bates, S.H. & Myers, M.G. Jr.: Trends Endocrinol Metab. 14:447-52, 2003
- Myers, M.G. Jr.: Recent Prog. Horm. Res. 59:287-304, 2004

## ELISA OVERVIEW

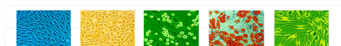
Cell Applications ELISA Kits are based on standard sandwich enzyme-linked immunosorbent assay technology. Freshly prepared standards, samples, and solutions are recommended for best results.

1. Prepare test samples.
2. Prepare a protein standard of the target protein.
3. Add test samples and standards to the pre-coated 96-well plate. Do not wash.
4. Add biotinylated detection antibodies. Wash.
5. Add Avidin-Biotin-Peroxidase Complex (ABC) Solution. Wash.
6. Add Tetramethylbenzidine (TMB) Color Developing Agent, containing HRP substrate.
7. Add TMB Stop Solution
8. Subject the plate to analysis.

### NOTES:

- Before using the kit, quick spin tubes to bring down all solution to the bottom of tube.
- Duplicate assay wells are recommended for both standard and sample testing.
- Do not let the 96-well plate dry, this will lead to inactivation of plate components.
- When diluting samples and reagents, ensure that they are mixed completely and evenly.
- Pre-warm diluted ABC and TMB solutions at 37°C for 30 min before use to avoid variable temperature effects.
- For washes, use TBS or PBS. Do not touch well walls.
- A protein standard is included in the kit. A protein standard detection curve should be generated with each experiment, no more than 2 hours prior to the experiment.
- The user will determine sample dilution fold by estimation of target protein amount in samples.

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## ELISA PROTOCOL

### Preparation of Test Samples

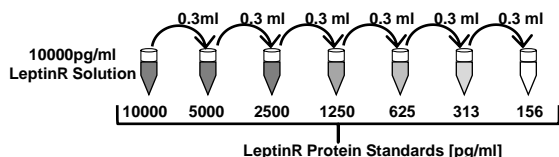
- Process Test Samples in the following manner:
  - Cell culture supernate, tissue lysate or body fluids:** Remove particulates by centrifugation.
  - Serum:** Allow the serum to clot in a serum separator tube (about 2 hours) at room temperature. Centrifuge at approximately 1000 x g for 15 min. Analyze the serum immediately or aliquot and store frozen at -20°C.
  - Plasma:** Collect plasma using heparin/EDTA/citrate as an anticoagulant. Centrifuge for 15 min at 1000 x g within 30 min of collection. Analyze immediately or aliquot and store frozen at -20° C.
- Estimate the concentration of the target protein in the sample and select a proper dilution factor such that the diluted target protein concentration falls within the 156-10000 pg/ml standard curve range. Depending on the sample, several trial dilutions may be necessary. Dilute the sample using the provided diluent buffer, mixing well. Suggested working dilutions of samples are as follows:

| Target Protein Concentration Range | Sample Working Dilution | Sample Vol. | Diluent Buffer Vol. |
|------------------------------------|-------------------------|-------------|---------------------|
| 100-1000 ng/ml                     | 1:100                   | 1 µl        | 99 µl               |
| 10-100 ng/ml                       | 1:10                    | 10 µl       | 90 µl               |
| 156-10000 pg/ml                    | 1:2                     | 50 µl       | 50 µl               |
| ≤156pg/ml                          | n/a                     | 100µl       | n/a                 |

- If samples will be assayed within 24 hours, store at 2-8° C. For long-term storage, aliquot and freeze samples at -20° C. Avoid repeated freeze-thaw cycles.

### Preparation of Standard Solutions (156-10000 pg/ml)

- Reconstitute the Lyophilized Recombinant Protein to make a 100.000 pg/ml human LeptinR solution. Add 1 ml Sample Diluent Buffer to a tube of lyophilized protein, keep the tube at room temperature for 10 min. Mix thoroughly.
- Add 0.1 ml of the mixed 10,000 pg/ml human LeptinR solution to the eppendorf tube containing 0.9 ml diluent buffer and mix to make a 1000 pg/ml human LeptinR solution.
- Label 6 eppendorf tubes with the LeptinR protein concentrations to be prepared by serial dilution: 5000pg/ml, 2500pg/ml, 1250pg/ml, 625pg/ml, 313pg/ml, 156pg/ml.
- Aliquot 0.3 ml of the Sample Diluent Buffer to the labeled tubes.
- Serially dilute the protein standards into their respectively labeled tubes. Transfer 0.3 ml from the 10000pg/ml LeptinR Solution to the 5000pg/ml eppendorf tube and mix thoroughly. Transfer 0.3 ml of the 5000 pg/ml solution to the 2500pg/ml tube and mix thoroughly, Transfer 0.3 ml of the 2500pg/ml solution to the 1250pg/ml tube and mix, and so on to make the 625, 313 and 156 pg/ml solutions.
- Store at 4° C until use.



### Loading the 96-well Plate

- Aliquot 0.1 ml of the sample diluent buffer into a control well to serve as the Blank. This will yield the O.D.450(Blank) reading.
- Aliquot 0.1 ml of the standard solutions of the **Preparation of Standard Solutions** (156-10000pg/ml) into empty wells of the precoated 96-well plate. Duplicate measurements of standards are recommended.

- Aliquot 0.1 ml of each properly diluted test sample to empty wells prepared in **Step 2**. Duplicate measurements of each test sample are recommended.
- Cover the 96-well plate and incubate at 37° C for 90 min.
- During the **Step 13** incubation period, prepare a stock of Biotinylated 1:100 Antibody Working Solution. Count the number of reactions and multiply by 0.1 ml/well for the Working Solution total volume (preparation of 1-2 reactions in excess of the number of wells is recommended). Dilute the Biotinylated Antibody to 1:100 in Antibody Diluent Buffer and mix thoroughly. Use the working solution within 2 hours.
- Upon completion of the 90 min incubation of **Step 13**, remove the cover of the 96 well plate and discard plate well contents. Blot the plate onto paper towels or other absorbent material. DO NOT let the wells completely dry at any time.
- Add 0.1 ml of the Biotinylated 1:100 Antibody Working Solution (prepared in **Step 14**) to each well and incubate the plate at 37° C for 60 min.
- During the incubation period of **Step 16**, prepare a stock of ABC Working Solution. Count the number of reactions and multiply by 0.1 ml/well for the Working Solution total volume (preparation of 1-2 reactions in excess of the number of wells is recommended). Dilute the ABC Stock Solution to 1:100 in ABC Diluent Buffer and mix thoroughly. Pre-warm the ABC working solution at 37° C for 30 min before use. Use the working solution within 1 hour.
- Upon completion of the 60 min incubation of **Step 16**, wash the plate 3 times with 0.3 ml TBS or PBS. For each wash, leave washing buffer in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
- Add 0.1 ml of prepared ABC Working Solution (prepared in **Step 17**) to each well and incubate the plate at 37° C for 30 min.
- During the incubation period of **Step 19**, pre-warm TMB Color Developing Agent at 37° C for 30 min before use.
- Upon completion of the 30 min incubation of **Step 19**, wash the plate 5 times with 0.3 ml TBS or PBS. For each wash, leave the washing buffer in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
- Add 90 µl of the pre-warmed TMB Color Developing Agent into each well and incubate at 37° C for 8-12 min (shades of blue can be seen in the wells with the four most concentrated Protein Standard Solutions; the other control wells should show no obvious color).
- Add 0.1 ml of the TMB Stop Solution to each well. The acidic stop solution will change the mixture color to yellow. The yellow intensity is proportional to the amount of target protein captured by the plate.
- Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution. These readings are the O.D.450(Reading).

### Calculating Protein Concentration

- For all wells, determine O.D.450(Relative):  

$$\text{O.D.450(Relative)} = \text{O.D.450(Reading)} - \text{O.D.450(Blank)}$$
- Plot the standard curve: plot O.D.450(Relative) of each standard solution (Y) vs. the respective concentration of the standard solution (X). See **Figure 1** for a typical standard curve.
- The human LeptinR concentration of the samples can be interpolated from the standard curve. Multiply the interpolated concentration by the dilution factor to obtain the target protein concentration in the sample.

