

Orexin-A ELISA

Cat. No: KBI5025
Ver1.0

Immunoassay for quantitative estimation of Orexin-A in
human serum, plasma and tissue extracts.

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Introduction:

Orexin-A, also known as hypocretin-1, is a naturally occurring neuropeptide and orexin isoform. The orexinergic nucleus in the lateral hypothalamus is the primary orexin projection system in the brain. Orexin-A is a peptide composed of 33 amino acids including an N-terminal pyroglutamyl residue and two intramolecular disulfide bridges between cysteine residues in 6 and 12 and 7 and 14 positions.

Intended Use:

The Orexin-A ELISA is used for estimation of Orexin-A in serum, plasma, or tissue extracts in pharmacokinetics, peptide delivery study and other purposes.

Principle:

The Orexin-A ELISA is a competitive immunoassay for the determination of Orexin-A. The antiserum is captured by antibodies coated on a 96-well plate. A constant concentration of Biotin Concentrate and varying concentrations of unlabeled standard or sample compete for binding specifically to the antiserum. Captured Bt-tracer is subsequently bound by SA-HRP (streptavidin-conjugated horseradish peroxidase), which produces a soluble colored product after a substrate is added. The enzyme reaction is stopped by dispensing an acidic solution (H₂SO₄) into the wells after 10 min at room temperature turning the solution from blue to yellow. The optical density (OD) of the solution at 450 nm is inversely proportional to the amount of specific Orexin-A bound.

Materials Provided:

1. Microtitre coated plate (96 wells) – 1 no
2. Biotin Concentrate (lyophilized) – 1 vial
3. Orexin-A Standard (lyophilized) - 1 vial
4. Standard Diluent - 1 bottle
5. Assay Buffer (20X), 50ml -1 bottle
6. Orexin-A Antiserum (lyophilized) - 1 bottle
7. Streptavidin-HRP (200X), 100 ul - 1 vial
8. TMB Substrate, 11ml – 1 bottle
9. Stop Solution, 15ml – 1 bottle
10. Instruction Manual

Materials to be provided by the End-User:

1. Microplate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes to measure volumes ranging from 50µl to 1000µl.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Timer.
6. Absorbent paper
7. Test tubes, pipettes and various other standard laboratory items

8. Extraction Kit (optional, recommended) Catalog No#KBI9010ext

Handling/Storage:

1. Reconstitute or dilute only the specific reagents mentioned in the reagent preparation section, when ready to run the assay.
2. After receiving the kit, store the lyophilized components at a constant -20°C for up to one year from the kit's expiration date. The remaining components should be stored in the refrigerator (2- 4°C) up to the kit's expiration date.
3. Do not use kit components after the expiration date.
4. Do not repeatedly freeze/thaw the reagents as loss of activity may result.
5. Before using, bring all components to Room Temperature (18-25°C). Upon assay completion return all components to appropriate storage conditions.
6. ELISA plate pouches contain dessicant. Keep the plates sealed in the pouch with dessicant in the refrigerator when not in use.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
2. Handle Stop Solution carefully. Obtain medical attention in case of accidental ingestion of kit components.
3. Avoid assay of samples containing Sodium azide as it is hazardous.

Reagent Preparation (all reagents should be diluted immediately prior to use):

1. 1X Assay Buffer: To make 1X Assay Buffer, add 50 ml of 20X Assay Buffer to 950ml of DI water. This is the working solution.
2. Orexin-A Antiserum: Add 5 ml of Assay Buffer and vortex.
3. Biotin Concentrate: Add 5 ml of Assay Buffer to the vial of lyophilized biotinylated peptide and vortex.
4. Standard: Prepare the standards using the schematic provided below using the Standard Diluent provided in the Kit.

Suggested Preparation of Standards		
	ng/ml	Range: 0.01 to 1000 ng/ml
Stock	1000	
S5	100	Add 20 ul stock + 180 ul Standard Diluent
S4	10	Add 20 ul S5 + 180 Standard Diluent
S3	1	Add 20 ul S4 + 180 Standard Diluent
S2	0.1	Add 20 ul S3 + 180 Standard Diluent
S1	0	500 ul Standard Diluent

Specimen Collection and Preparation:

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

Serum: Use a serum separator tube and allow clotting for 30 minutes, then centrifuge for 10 minutes at 1000 x g. Remove serum layer and assay immediately or store serum samples at < -20°C. Avoid repeated freeze/thaw cycles.

Dilute the serum 1:50 in Sample Diluent. For example: add 5 ul of serum sample to 245 ul of 1X Sample Diluent. If not assayed immediately, diluted samples should be stored at -20°C or below.

Sample Extraction. Factors present in serum may bind to the antiserum complex. The effects can vary from negligible to complete obliteration of signal. Sample extraction may, therefore, be required prior to using the kit. It is recommended to use the KRISHGEN Extraction Kit (optional, recommended) Catalog No#KBI9010ext. The kit may work for some tissue culture samples without the extraction. See "Protocol for Sample Extraction" in the data sheet / kit insert / IFU of Catalog No.#KBI9010ext. The kit may still be used without extraction but this may cause unexpected results due to the possible binding between serum proteins and kit components.

Procedural Notes:

1. Read all the instructions thoroughly before performing the test.
2. Allow all reagents to reach Room Temperature before beginning and reconstitute or dilute the required reagents.
3. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess unreacted reagents is essential.
4. All Standards, Controls and Samples should be assayed at least in duplicates.
5. The assay has been optimized to be used with the protocol mentioned. Any deviation from the same may invalidate the results.

Assay Procedure:

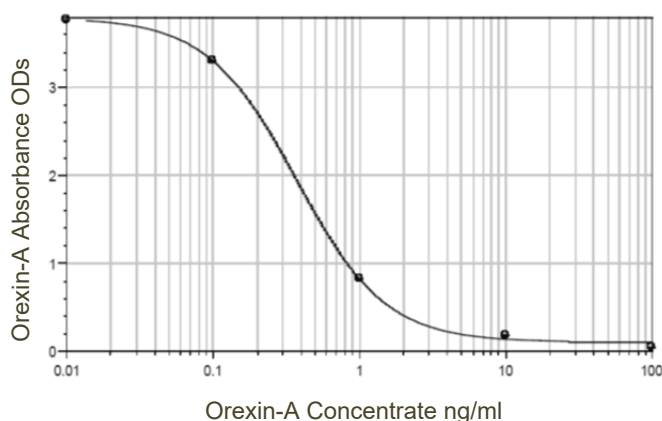
1. Bring all reagents to Room Temperature prior to use. It is strongly recommended that all **Controls and Samples** should be run in duplicates or triplicates.
2. Pipette 50 ul **Standard** or **Sample** (in Assay Buffer) and 25 ul **Orexin-A Antiserum** (in Assay Buffer) into each respective microwell except the blank microwells.
3. Add 75 ul **Assay Buffer** to the blank microwells.
4. Pipette 25 ul of the reconstituted **Biotin Concentrate** into each microwell.
5. Aspirate and wash plate 5 times with **Assay Buffer** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly or alternatively a microtiter plate or strip washer may be used.
6. Add 100 ul of freshly prepared **Streptavidin-HRP**. *Tap or centrifuge the SA-HRP vial to collect all liquid contents on the bottom of the vial. Dilute 1/200 in Assay Buffer (60 ul / 12 ml) and vortex. Add 100 ul to all wells, including the blanks.*
7. Incubate at Room Temperature for 60 minutes.
8. Wash the plate as per the Procedure given in step 5 above.
9. Add 100 ul of **TMB Substrate** into each microwell of the plate.
10. Cover the plate with a parafilm and incubate at Room Temperature under dark for 30-60 minutes.
11. Add 100 ul of **Stop Solution** to each microwell.

12. Measure the optical density of the wells on a plate reader at 450 nm within 10 minutes.

Interpretation of the Results:

Plot data and calculate results. We recommend that you use curve fitting software for your data analysis. Plate readers often include such software packages. This is, however, not essential and you may opt to plot manually on semi-log paper. You can also use a spreadsheet program.

We recommend log / lin processing for best results. The standard curve is established by plotting the mean OD-values of the calibrators on the ordinate, y-axis, (lin. scale) versus their respective Orexin-A concentrations on the abscissa, x-axis, (log. scale). Orexin-A concentrations of the unknown samples are directly read off in ng/ml against the respective OD values.



Precautions:

1. Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this set.
2. Substrate is light and heat sensitive hence do not expose it to direct sunlight while pipetting or incubating.
3. Samples and kit reagents after use should be disposed off observing appropriate regulations.
4. If necessary it is recommended that the results should be confirmed by an alternative method.
5. Do not dilute or adulterate test reagents or use samples not called for in the test procedure.

Performance Characteristics:

Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

1. Specificity:

The antibodies in this kit are specific for Orexin-A peptide. The standard used is a synthetic peptide for Orexin-A.

Synthetic Peptide Sequence: Pyroglu-Pro-Leu-Pro-Asp-Cys-Cys-Arg-Gln-Lys-Thr-Cys-Ser-Cys-Arg-Leu-Tyr-Glu-Leu-Leu-His-Gly-Ala-Gly-Asn-His-Ala-Ala-Gly-Ile-Leu-Thr-Leu

2. Cross Reactivity:

Orexin-A:	100%
Somatostatin:	0%

3. Precision:

The kit's IC₅₀, or the shape of the standard curve, may exhibit some variation but this will not affect the kit's accuracy in the measuring range. The kit accurately measures sample peptides if the following conditions are met.

- i) Both samples and standards must be measured in the same diluent and under the same conditions.
- ii) The kit's antibodies must not cross-react appreciably with other factors present in the sample. The user may wish to test the cross-reactivity with other peptides.
- iii) The sample peptides must be identical to the standard. Ideally the synthetic standard mimics the natural peptide perfectly. Sometimes, however, natural peptides exist as families of species related by a common or similar sequence. In addition, natural peptides may be enzymatically or spontaneously modified, may exist in complexes, and may assume alternative structural forms. In these cases the kit might not measure the exact concentration of a particular natural peptide species, but, it may still be used for relative average measurements.
- iv) Sample extraction. Factors present in serum can bind to kit components. The effects can vary from negligible to complete obliteration of signal. Sample extraction may, therefore, be required prior to using the kit.

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