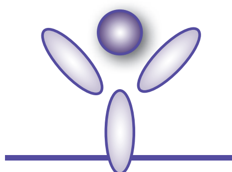
LEGEND MAX™

ELISA Kit



Human α-Synuclein Aggregate

Cat. No. 448807

ELISA Kit for Accurate Quantitation of
Human α-Synuclein Aggregate
in Cerebrospinal Fluid and Tissue Lysate

BioLegend, Inc.
biolegend.com

It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.

For Research Purposes Only. Not for use in diagnostic or therapeutic procedures. Purchase does not include or carry the right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of BioLegend is strictly prohibited.

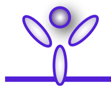


Table of Contents — Page

Introduction.......... 2

Materials Provided.......... 2

Materials to be Provided by the End-User.......... 3

Storage Information.......... 3

Health Hazard Warnings.......... 3

Specimen Collection and Handling.......... 4

Reagent and Sample Preparation.......... 4

Assay Procedure.......... 5

Assay Procedure Summary.......... 7

Calculation of Results.......... 8

Typical Data.......... 8

Performance Characteristics.......... 9

- Specificity.......... 9
- Sensitivity.......... 9
- Recovery.......... 9
- Linearity.......... 9
- Intra-Assay Precision.......... 10
- Inter-Assay Precision.......... 10
- Biological Samples.......... 10

Troubleshooting Guide.......... 11

ELISA Plate Template.......... 13

Introduction:

α-Synuclein is a neuronal protein which has a critical role in regulating the trafficking of pre-synaptic vesicles through interaction with the SNARE complex. In healthy neurons, α-Synuclein exists mainly as a monomeric protein; however, within a diseased cell, α-Synuclein aggregates to form insoluble fibril which is toxic to the cell. In fact, the aggregated form of the α-Synuclein is the major constituent of the Lewy body inclusion, which is the hallmark of Parkinson's disease, dementia with Lewy body, and other α-Synuclein-related neurodegenerative diseases. The formation and accumulation of α-Synuclein aggregate could impair many cellular processes from DNA repair to the release of neurotransmitters. Detection of α-Synuclein aggregate in the cerebrospinal fluid has been shown to have the potential to enhance the diagnosis of Parkinson's disease and other α-Synuclein associated disorders.

The LEGEND MAX™ Human α-Synuclein Aggregate ELISA Kit is a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with a 96-well strip plate that is pre-coated with anti-human α-Synuclein Aggregate antibody. The detection antibody is a biotinylated anti-human α-Synuclein monoclonal antibody. This kit is specifically designed for the accurate quantitation of human α-Synuclein Aggregate from cerebrospinal fluid and tissue lysate. It is analytically validated with ready-to-use reagents.

Materials Provided:

Description	Quantity	Volume	Part #
Human α-Synuclein Aggregate pre-coated 96-well Strip Microplate	1 plate		750002622
Human α-Synuclein Aggregate Detection Antibody	1 bottle	12 mL	750002624
Human α-Synuclein Aggregate Lyophilized Standard	1 vial	lyophilized	750002627
Streptavidin-Polymer HRP	1 bottle	12 mL	750002513
2X Reagent Diluent	1 bottle	32 mL	76457
Wash Buffer (20x)	1 bottle	50 mL	78233
Substrate Solution F	1 bottle	12 mL	79132
Stop Solution	1 bottle	12 mL	79133
Plate Sealers	4 sheets		78101

Materials to be Provided by the End-User:

- Microplate reader able to measure absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 1 µL to 1,000 µL
- Deionized water
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Polypropylene tubes to prepare standard dilutions
- Timer
- Plate Shaker
- Polypropylene vials

Storage Information:

Store unopened kit components between 2°C and 8°C. Do not use this kit beyond its expiration date.

<table>
<tr><th colspan="2">Opened or Reconstituted Components</th></tr>
<tr><td>Microplate wells</td><td>If not all microplate strips are used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal. Store between 2°C and 8°C for up to one month.</td></tr>
<tr><td>Standard</td><td>The remaining reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Streptavidin-Polymer HRP</td><td rowspan="5">Store opened reagent bottles at 2° - 8°C and use within 1 month</td></tr>
<tr><td>2X Reagent Diluent</td></tr>
<tr><td>Wash Buffer (20X)</td></tr>
<tr><td>Substrate Solution F</td></tr>
<tr><td>Stop Solution</td></tr>
</table>

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online at BioLegend's website for details (www.biolegend.com/msds).
2. Substrate Solution F is harmful if inhaled or ingested. Avoid skin, eye and clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents,

handle all serum, plasma and other biological fluids in accordance with NCCLS regulations.

4. Stop Solution contains strong acid. *Wear eye, hand, and face protection.*
5. Before disposing of the plate, rinse it with an excess amount of tap water.

Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. If possible, unknown samples should be run at a number of dilutions to determine the optimal dilution factor that will ensure accurate quantitation.

Cerebrospinal Fluid: If necessary, centrifuge all samples to remove debris prior to analysis. Store samples at < -70°C. Avoid repeated freeze-thaw cycles.

Tissue lysate: Rinse and then homogenize tissue in PBS. Add an equal volume of NP40 lysis buffer (50 mM Tris-HCL, 150 mM NaCl, 1% NP-40) containing protease inhibitor and lyse tissue at room temperature for 30 min with gentle agitation. Centrifuge to remove debris, then assay immediately or store at <-70°C. Quantify total protein using a total protein assay.

Reagent and Sample Preparation:

Note: All reagents should be diluted immediately prior to use.

1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water. If crystals have formed in the 20X Wash Buffer, bring to room temperature and vortex until dissolved.

2. Reagent Diluent (1X): Dilute the 2X Reagent Diluent to 1X with deionized water. For example, make 40 mL of Reagent Diluent (1X) by adding 20 mL of 2X Reagent Diluent to 20 mL of deionized water.

3. Reconstitute the lyophilized Human α-Synuclein Aggregate Standard by adding the volume of Reagent Diluent (1X) to make the 10 ng/mL standard stock solution (Refer to LEGEND MAX Kit Lot-Specific Certificate of Analysis/ LEGEND MAX Kit Protocol). Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.

4. In general, no dilution is needed for Human Cerebrospinal Fluid samples. It is recommended to dilute Human Brain Lysate samples in Reagent Diluent (1X) down to a total protein concentration of 50 µg/mL. However, samples can be diluted further to fit within the range of the assay as determined by the end user.

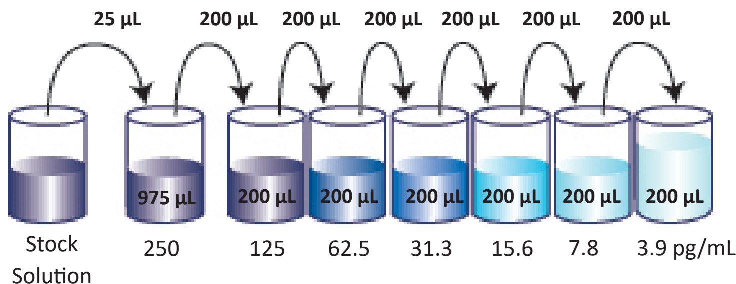
Assay Procedure:

Note: Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this kit.

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.

2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.

3. Prepare 1000 µL of the 250 pg/mL top standard by adding 25 µL of the 10 ng/mL standard stock solution into 975 µL Reagent Diluent (1X). Perform six two-fold serial dilutions of the 250 pg/mL top standard in separate tubes using Reagent Diluent (1X) as the diluent. Thus, the α-Synuclein Aggregate standard concentrations in the tubes are 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL and 3.9 pg/mL, respectively. Reagent Diluent (1X) serves as the zero standard (0 pg/mL).



4. Wash the plate 4 times with at least 300 µL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes should be performed similarly.

5. Add 50 µL of Reagent Diluent (1X) to each well that will contain either standard dilutions or samples. Then add 50 µL of standard dilutions or samples to the appropriate wells.

6. Seal the plate with a Plate Sealer included in the kit and incubate the plate for 2 hours at room temperature with shaking.

7. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

8. Add 100 µL of Human α-Synuclein Aggregate Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour while shaking.

9. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

10. Add 100 µL of Streptavidin-Polymer HRP solution to each well, seal the plate and incubate at room temperature for 30 minutes while shaking.

11. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 4. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

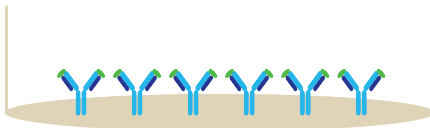
12. Add 100 µL of Substrate Solution F to each well and incubate for 20 minutes in the dark. Wells containing Human α-Synuclein Aggregate should turn blue in color with an intensity proportional to its concentration. It is not necessary to seal the plate during this step.

13. Stop the reaction by adding 100 µL of Stop Solution to each well. The solution color should change from blue to yellow.

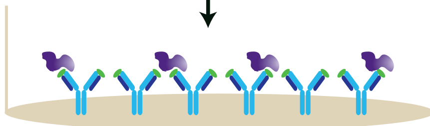
14. Read absorbance at 450 nm within 30 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

Assay Procedure Summary

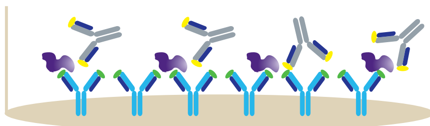
1. Wash 4 times.
   Add 50 µL Reagent Diluent (1X) to standard wells and sample wells.



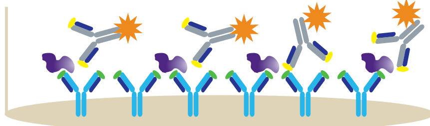
2. Add 50 µL of standard or sample. Incubate 2 hr, RT, shaking.



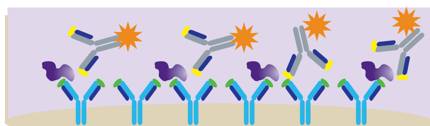
3. Wash 4 times.
   Add 100 µL of Human α-Synuclein Aggregate Detection Antibody solution.
   Incubate 1 hr, RT, shaking.



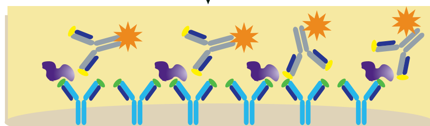
4. Wash 4 times.
   Add 100 µL Streptavidin-Polymer HRP solution.
   Incubate 30 min, RT, shaking.



5. Wash 5 times.
   Add 100 µL Substrate Solution F.
   Incubate 20 min, RT, in the dark.



6. Add 100 µL Stop Solution.



7. Read absorbance at 450 nm and 570 nm.

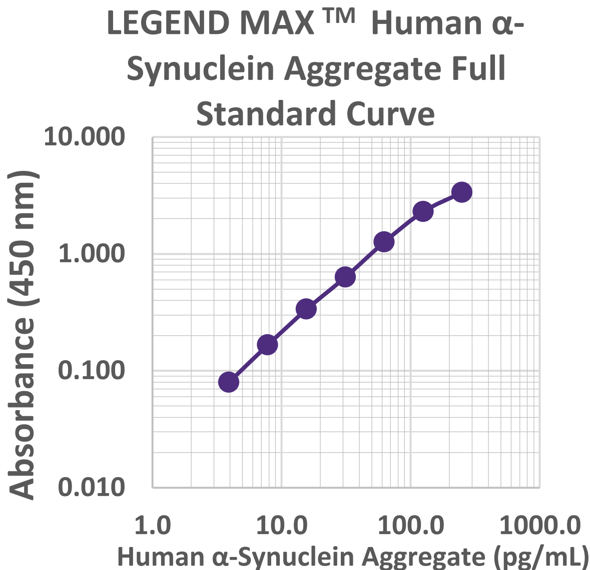
Calculation of Results:

The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If an appropriate software is not available, use log-log graph paper to determine sample concentrations. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with analyte concentration on the X-axis and absorbance on the Y-axis. Draw a best fit line through the standard points. To determine the unknown analyte concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the analyte concentration.

If samples were diluted, multiply the concentration by the appropriate dilution factor. If a test sample's absorbance value falls outside the linear portion of the standard curve, the test sample needs to be re-analyzed at a higher (or lower) dilution as appropriate.

Typical Data:

This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



Performance Characteristics:

Specificity: This kit recognizes Human α-Synuclein Aggregate. No significant cross reactivity was observed when this kit was used to analyze the following recombinant proteins at 2500 pg/mL.

Human	α-Synuclein monomer	β-Synuclein monomer	γ-Synuclein monomer
% cross reactivity	0.105%	0%	0%

Sensitivity: The minimum detectable concentration of Human α-Synuclein Aggregate is 0.74 ± 0.19 pg/mL (n=6).

Recovery: Recombinant human α-Synuclein Aggregate at 3 different concentrations was spiked into different samples of Human Cerebrospinal Fluid, Human Brain Lysate, and the NP40 Lysis Buffer. Sample recovery was then analyzed with the LEGEND MAX™ Human α-Synuclein Aggregate kit.

Sample Type	N	% Recovery
Human Cerebrospinal Fluid	4	93.5%
Human Brain Lysate	2	112.5%
NP40 Lysis Buffer	1	98.0%

Linearity: Neat natural Human Cerebrospinal Fluid samples and neat NP40 Lysis Buffer were spiked with α-Synuclein Aggregate. Human Brain Lysate samples were diluted down to 100 µg/mL total protein. Then, they were diluted 2 fold in serial to produce samples within the dynamic range of the kit. Samples were then assayed to determine the dilutional linearity.

Sample Type	N	% Linearity
Human Cerebrospinal Fluid	4	102.0%
Human Brain Lysate	2	108.5%
NP40 Lysis Buffer	1	116.0%

Intra-Assay Precision: Two samples containing different human α-Synuclein Aggregate concentrations were tested on one plate with 12 replicates.

Concentration	Sample 1	Sample 2
Number of Replicates	12	12
Mean Concentration (pg/mL)	121	23
Standard Deviation	4.0	0.8
%CV	3%	4%

Inter-Assay Precision: Two samples containing different human α-Synuclein Aggregate concentrations were tested in ten independent assays.

Concentration	Sample 1	Sample 2
Number of Assays	10	10
Mean Concentration (pg/mL)	107	19
Standard Deviation	9.4	2.9
%CV	9%	15%

Biological Samples: Human Cerebrospinal Fluid samples were assayed for natural α-Synuclein Aggregate. Two Human Brain Lysate samples were diluted down to 50 µg/mL of total protein and then assayed for α-Synuclein Aggregate.

	Cerebrospinal Fluid	Human Brain Lysate
N	24	2
Min (pg/mL)	ND	3.9
Max (pg/mL)	77.2	13.3
Mean (pg/mL)	6.0	8.6
% Detectable	33%	100%
average pg α-Synuclein Aggregate / µg total protein	NA	0.172

Troubleshooting Guide:

Problem	Probable Cause	Solution
High Background	Background wells were contaminated	Avoid cross-well contamination by using the provided plate sealers. Use multichannel pipettes and change tips between pipetting samples and reagents.
	Insufficient washes	Increase number of washes. Increase soaking time between washes prior to addition of substrate solution.
	TMB Substrate Solution was contaminated	TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting substrate solution into wells.
No or poor signal	Detection Antibody, Avidin-HRP or Substrate solution were NOT added	Rerun the assay and follow the protocol.
	Wrong reagent or reagents were added in wrong sequential order	
	Insufficient plate agitation	The plate should be agitated during all incubation steps using a plate shaker at a speed where solutions in wells are within constant motion without splashing.
	The wash buffer contains Sodium Azide (NaN3)	Avoid Sodium Azide contamination in the wash buffer as it inhibits HRP activity.
	Incubations were done at an inappropriate temperature, timing or without agitation	Rerun the assay and follow the protocol.
Low or poor standard curve signal	The standard was incorrectly reconstituted or diluted	Adjust the calculations and follow the protocol.
	Standard was inappropriately stored	Store the reconstituted standard stock solution in polypropylene vials at -70°C. Avoid repeated freeze-thaw cycles.
	Reagents added to wells with incorrect concentrations	Check for pipetting errors and the correct reagent volume.

Problem	Probable Cause	Solution
Signal is high, standard curves have saturated signal	Standard reconstituted with less volume than required	Reconstitute new lyophilized standard with the correct volume of solution recommended in the protocol.
	Standards/samples, detection antibody, Avidin-HRP or substrate solution were incubated for too long	Rerun the assay and follow the protocol.
Sample readings are out of range	Samples contain no or below detectable levels of the analyte	If samples are below detectable levels, it may be possible to use a larger sample volume. Contact technical support for appropriate protocol modifications.
	Samples contain analyte concentrations greater than highest standard point	Samples may require dilution and analysis.
High variation in samples and/or standards	Multichannel pipette errors	Confirm that pipette calibrations are accurate.
	Plate washing was not adequate or uniform	Ensure pipette tips are tightly secured. Ensure uniformity in all wash steps.
	Non-homogenous samples	Thoroughly mix samples before assaying.
	Samples may have high particulate matter	Remove particulate matter by centrifugation.
	Cross-well contamination	Do not reuse plate sealers. Always change tips for reagent additions. Ensure that pipette tips do not touch the reagents on the plate.

Tel: 858-768-5800

ELISA Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

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