α-Synuclein ELISA Kit
Instruction Manual

Catalog # NS400

Sufficient reagents for 96 assays per kit

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.

upstate | CHEMICON | Linco

THE EXPERTISE OF UPSTATE®, CHEMICON® AND LINCO®
IS NOW A PART OF MILLIPORE

USA & Canada Phone: +1(800) 437-7500 Fax: +1 (951) 676-9209
http://www.millipore.com

Background

α-Synuclein, a member of the synuclein family which also includes β- and γ-synuclein, is a small cytoplasmic protein predominately expressed in neural tissue, particularly in the neocortex, hippocampus, striatum, thalamus, and cerebellum. Though its normal physiological function is unclear, α-Synuclein is highly enriched at the presynaptic terminals and may have a role in membrane associated processes at the presynaptic terminal. It is specifically up regulated in presynaptic terminals of discrete neuronal populations in songbird brain during a period of song-acquisition-related synaptic rearrangement[1]. Mice deficient in α-Synuclein display enhanced dopamine release in the nigrostriatal dopamine system suggesting that α-synuclein is a negative regulator of dopamine neurotransmission[2]. Furthermore, knockdown of α-synuclein in cultured hippocampal neurons results in a decline in the distal pool of presynaptic vesicles[3]. Also, purified α-Synuclein inhibits phospholipase D2 mediated hydrolysis of phosphatidylcholine to phosphatidic acid[4] which may play a role in cytoskeletal reorganization and endocytosis at the plasma membrane[5]. Thus, α-Synuclein may serve to integrate presynaptic signaling and membrane trafficking.

Dysfunctional regulation in α-Synuclein is strongly implicated as a critical factor in several neurodegenerative diseases. Misfolding, aggregation and fibrillation are associated with the pathogenesis of Parkinson disease[6]. α-Synuclein peptides are also a major component of amyloid plaques in Alzheimer's disease[7]. It has also been identified in the Glial Cytoplasmic Inclusions found in Multiple System Atrophy[8].

Test Principle

The α-Synuclein ELISA kit is a solid phase sandwich enzyme linked immunosorbent assay that provides a fast, sensitive method to detect specific levels of α-Synuclein protein in whole cell or tissue extracts (Figure 1). The kit utilizes a 96-well microtiter plate that is pre-coated with an α-Synuclein specific capture antibody. A test sample or the protein standard provided in the kit is then added to each test well and incubated allowing for α-Synuclein antigen to be captured. The plate is subsequently washed to remove any unbound non-specific material and then incubated with a detection antibody that is specific to α-Synuclein. Any unbound detection antibody is washed away prior to a final incubation with HRP conjugated secondary antibody. After the addition of TMB substrate and stop solution, the absorbance is measured at 450 nm using a plate reader. This enzymatic detection format allows for sensitive and specific detection of α-Synuclein protein in test samples.

The entire assay takes less than 5 hours to complete with minimal hands-on time. For ease of use many of the reagents are supplied in a ready-to-use formulation. The kit also includes an α-Synuclein protein standard that is run as a positive control to develop a standard curve.

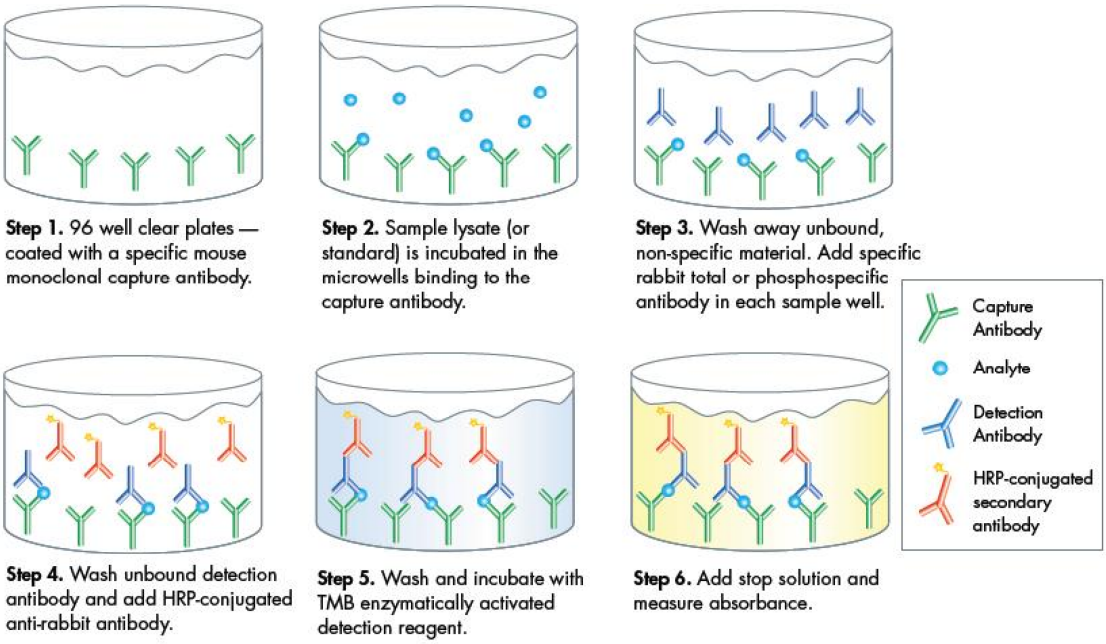


Figure 1: Schematic of sandwich enzyme linked immunosorbent assay.

Analytical Sensitivity, Detection Limits, and Species Reactivity

Sensitivity:	3 ng/ml
Range of Detection:	3 to 60 ng/ml
Species Reactivity:	Human, mouse and Rat. Have not tested other species.

***NOTE**: This data is presented for reference use only and should not be used to interpret actual assay results. A standard curve must be generated for each assay.*

Storage & Stability

Storage: The α-Synuclein ELISA Kit is a dual temperature storage kit. Upon receipt, the α-Synuclein protein standard and anti-α-Synuclein Detection Antibody (NS400-2) should be stored at -20ºC. All other kit components (NS400-1) should be stored at 4ºC.

Stability: Kit components are stable for a minimum of 4 months from the date of receipt if stored and handled as described above.

Kit Components

NS400-1 (Store at 2-8℃)

- 96-well α-Synuclein Assay Plate: One 96 well plate.
- 5X Lysis Buffer/ELISA Diluent: One 20ml bottle.
- 10X Wash Buffer: One 130ml bottle.
- TMB Solution: One 10ml bottle.
- Stop Solution: One 12ml bottle.
- HRP Conjugated Secondary Antibody: One vial with 10µl.

NS400-2 (Store at -20℃)

- α-Synuclein Protein Standard: One vial with 10µl of 1mg/ml standard.
- Detection Antibody: anti-α-Synuclein: One vial with 10µl.

***NOTE:** The reagents supplied in this kit are sufficient to process one 96 well plate.*

Materials Not Supplied

1. Tween® 20
2. Protease Inhibitors
3. Multi-channel or repeating pipettes
4. Plate shaker/washer (optional)
5. Pipettors & tips capable of accurately measuring 1-1000 µL
6. Graduated serological pipettes
7. 96-well microtiter plate reader with 450 nm filter
8. Graphing software for plotting data or graph paper for manual plotting of data
9. Microfuge tubes for standard and sample dilutions
10. Mechanical vortex
11. MilliQ™ Water
12. 96 well plate sealing tape

Precautions

- This kit is for research use only; not for use in diagnostic procedures.
- The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and a failure to produce accurate data.
- Caustic Material: Stop Solution. The stop solution contains hydrochloric acid which is harmful if swallowed or inhaled; avoid contact with skin and eyes (wear gloves and eye protection); wash areas of contact immediately with water.

  Caution: Eye, hand, face, and clothing protection should be worn when handling this material.
- **Safety Warnings and Precautions:** This kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.
- The Detection Antibody and 5X Lysis Buffer / ELISA Diluent contain sodium azide. Sodium azide may react with copper and lead plumbing to form highly explosive metal azides. Upon disposal, flush with large amounts of water to prevent azide build-up. Avoid contact with skin.

Technical Notes

- The TMB Solution should be warmed to room temperature (20°C to 25°C) just prior to use.
- Manual plate washing: Vigorous washing and complete removal of all liquid by aspiration at the end of each washing step is very important to obtain low background values. Gentle agitation during the wash steps or a 2-3 minute soak may reduce background values.
- Automatic plate washing: Automatic plate washing is a convenient option provided the wash apparatus is properly maintained and operating correctly. Tubing and tips can easily become clogged, leading to incomplete washing and inadequate aspiration of wells. The result may be poor precision and an unsuitable standard curve. Please note that optimization of the protocol may be necessary when using a plate washer. For example, additional wash cycles may need to be added.
- The desiccant enclosed in the pouch for the 96-well plate will aid in stability should the plate lose its seal during shipping.

- The kit contains the recommended cell lysis buffer optimized to work in this assay. Though other lysis buffers can be used to make lysate (i.e. RIPA buffer), they must by diluted using the provided Lysis Buffer or ELISA Diluent (diluted to 1X) prior to use in the ELISA assay. Detergent amounts must be at or below the following levels in the ELISA assay: 0.001% Triton® X100, 0.005% CHAPS, 0.001% SDS, 0.0001% Nonidet® P40, and 0.000025% Deoxycholate.

Preparation of Reagents and Samples

Follow the directions below to prepare reagents and solutions necessary for performing the α-Synuclein ELISA Kit.

Wash Buffer: The Wash Buffer provided is supplied as a 10X concentrate without Tween® 20. Dilute the concentrate to 1X strength using Milli-Q™ water and add Tween® 20 to equal a final concentration of 0.05%. For example, add 897.5 ml of Milli-Q™ water to 100 ml of 10X wash buffer and 2.5 ml of 20% Tween® 20.

Lysis Buffer / ELISA Diluent: The Lysis Buffer / ELISA Diluent is provided as a 5X stock. To make a working stock of Lysis Buffer dilute the 5X stock to 1X using Milli-Q™ water and add protease inhibitors. A cocktail of protease inhibitors should be utilized to enhance protein stability. The 1X Lysis Buffer can also be made without protease inhibitors and used as an ELISA Diluent. Note that protease inhibitors can be added when the buffer is used as a diluent but they are generally not necessary.

10 mL of 1X Lysis Buffer containing protease inhibitors can be prepared by adding 10 µL of 1 µg/µL Leupeptin, 10 µL of 1 µg/µL Aprotinin, 10 µL of 1 µg/µL Pepstatin, 100 µL of freshly diluted 100mM PMSF, 2 mL of 5X Lysis Buffer to 7.87 mL of Milli-Q™ water.

Detection Antibody: Dilute the detection antibody 1:500 with 1X ELISA Diluent immediately before use. There is sufficient antibody for one 96-well plate.

HRP Conjugated Antibody: Dilute the enzyme conjugated antibody 1:5000 with 1X wash buffer immediately before use. There is sufficient antibody for one 96-well plate.

α-Synuclein Protein Standards:
Ten microliters of the α-Synuclein protein standard (at 1 mg/mL) is provided. Avoid repeated freeze/thawing of this material. If necessary, make aliquots and store them at -80°C until ready for use.
NOTE: *The following procedure will make enough standard to run in duplicate wells.*

1. Make a 3000 ng/ml stock solution by diluting 3 µl of the 1 mg/ml standard into 997 µl of 1X ELISA Diluent. Then make a 92.3 ng/ml working stock solution by diluting 30.8 µl of the 3000 ng/ml stock solution into 969.2 µl of 1X ELISA Diluent (total volume 1000 µl).
2. Label eight test tubes numbers “2 thru 8” and “0”. Add 280 µl of the 1X ELISA Diluent to tubes number “2 thru 8” and “0”.
3. Add 520 µL of the 92.3 ng/ml working stock solution of standard to tube #2 and vortex. Standard tube #2 will now have a concentration of 60 ng/mL of protein.
4. Perform a serial dilution by adding 520 µL of each preceding standard to tubes #3-8 (Refer to Figure 2). For example, to make standard #3, remove 520 µL of standard #2 and add it to tube #3 and vortex. Do not add any protein standard to the "0" or blank tube.

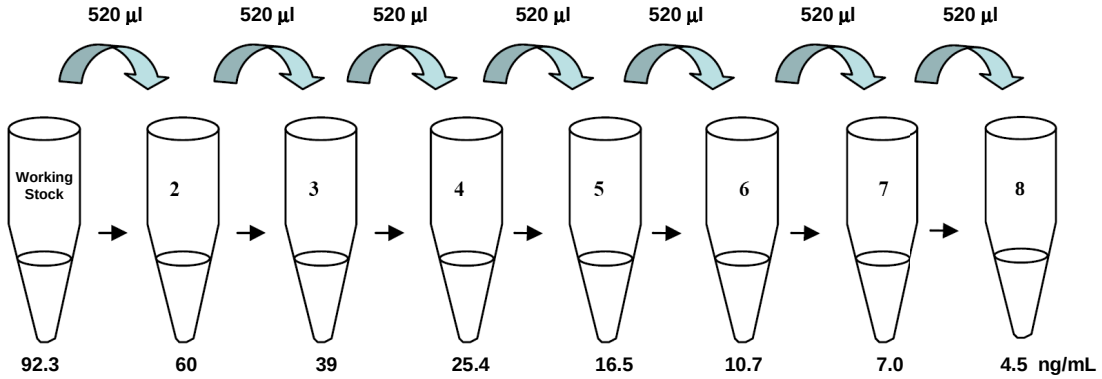


Figure 2: Schematic of serial dilutions and concentrations for preparation of standards.

Preparation of Cultured Cells:

Researchers should optimize the cell extraction procedures for their own applications. The following protocol has been successfully employed for several cell lines.

1. Culture cells using standard lab protocol.
2. Remove culture media and wash cells twice with PBS. Discard supernatant.
3. Add 5-10 mL of ice-cold 1X Lysis Buffer (containing protease inhibitors) per 150 mm tissue culture plate.
4. Scrape cells from plate with a rubber policeman.
5. Transfer the cell solution to a microcentrifuge tube and incubate on ice for 15 minutes.
6. Vortex tube for 10 seconds or sonicate briefly for 5-10 seconds.
7. Clarify lysate by centrifugation at 12,000 rpm for 10 minutes at 4ºC in a microcentrifuge prior to use.
8. Collect the supernatant and calculate protein concentration using a Bradford Assay or by densitometry.
9. Dilute the lysate with ELISA Diluent. The dilution factor requires adjustment depending on the quantity of α-Synuclein present. Researchers should determine the optimal dilution factor for each specific experimental condition. As a guideline, the lysate can be diluted to a final concentration of 10 -100 µg/mL.

Preparation of Tissue:

Researchers should optimize the tissue extraction procedure for their own applications. The following protocol has been successfully employed for several different tissue types.

1. Isolate tissue as desired. Transfer into a 50 ml tube and wash twice with PBS.
2. Add ice-cold 1X Lysis Buffer (containing protease inhibitors) to the tissue. The volume of Lysis Buffer to add requires adjustment depending on the quantity of tissue present. As a guideline, add 5-10x the wet mass of the tissue. Start by adding 1x the wet mass of the tissue (100 µl aliquots) and titrate with a pipettor to break up the tissue (if necessary a syringe may be used to shear the tissue). Add more Lysis Buffer as is necessary to achieve a homogenous solution. The solution can also be sonicated briefly to aid in the disruption of cells.

3. Clarify tissue lysate by centrifugation at 12,000 rpm for 10 minutes at 4ºC in a microcentrifuge prior to use.
4. Collect the supernatant and calculate protein concentration using a Bradford Assay or by densitometry.
5. Dilute the lysate with 1X ELISA Diluent. The dilution factor requires adjustment depending on the quantity of α-Synuclein present. Researchers should determine the optimal dilution factor for each specific experimental condition. As a guideline, the lysate can be diluted to a final concentration of 10 -100 µg/mL.

It is suggested that the cell lysate from tissue or cultured cells be used immediately following preparation. However, samples can be aliquoted and frozen at -80ºC for later use. For optimal results, frozen samples should be used within 6 months. Avoid repeated freeze thaws.

Further information regarding lysate preparation protocols can be obtained at *http://www.millipore.com*.

Assay Protocol

1. Prepare the reagents as described in the Reagent Preparation section.
2. Identify the number of reaction wells needed and remove any unneeded strip wells (8 wells per strip) from the plate holder. To do so carefully turn the plate upside down and push the strips from the bottom out through the plate. Re-seal the extra strips in the bag and store in the refrigerator for future use.
3. Add 100 µL of the zero dose ("0") into two wells for use as a negative control. Add 100 µL of diluted Standards number 2 through 8 as well as all test samples to additional wells of the plate. It is recommended that all standards and samples be run in at least duplicate.

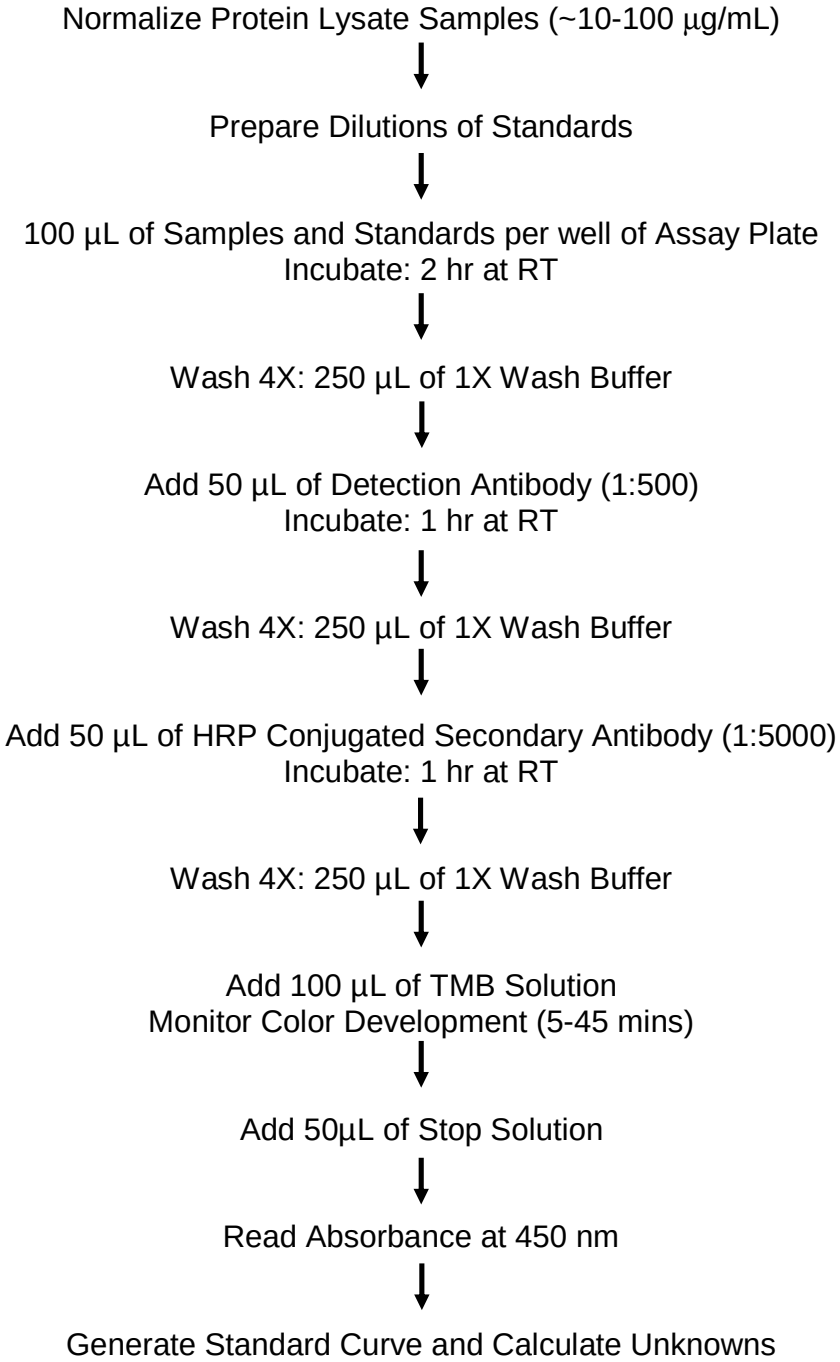
   Note: *A standard curve must be generated with each separate assay that is performed. Do not add standard or lysate to wells reserved for TMB blanks.*
4. Incubate the plate at room temperature for 2 hours with gentle shaking (this step can also go overnight at 4℃; seal plate with tape if incubatin g overnight).
5. Wash the plate with 250µL of 1X Wash Buffer per well. Repeat this at least 4 times.

   Note: Thorough washing of the plate is extremely important to reduce background. We recommend using a multi-channel pipette to fill each well with diluted Wash Buffer. Fluid removal from the wells is best accomplished by inverting the plate over a sink and flicking the fluid out of the wells and then blotting the plate on clean paper towels. If using an automated plate washer please see technical notes section for more information.
6. Add 50 µL of the detection antibody (pre-diluted 1:500 with 1X ELISA Diluent) to each well.
7. Incubate at room temperature for 1 hour with gentle shaking.
8. Wash the plate with 1X Wash Buffer at least 4 times and blot the wells dry as described above.
9. Add 50 µL of the HRP conjugated secondary antibody (pre-diluted 1:5000 with 1X Wash Buffer) to each well.
10. Incubate at room temperature for 1 hour with gentle shaking.
11. Wash the plate with 1X Wash Buffer at least 4 times and blot the wells dry as described above.

12. Add 100 µL of the TMB Solution to each well. Incubate at room temperature for 5 to 45 minutes, monitor the color development (see section A below).
13. Stop the reaction by adding 50 µL of Stop Solution to each well. Gently mix and then read the plate using a spectrophotometer set at 450 nm. Plate should be read within 1 hour of adding the stop solution. The plate reader may be blanked against a negative control well prepared by adding 50 µL of stop solution to 100 µL of the TMB solution.

NOTE: *Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the absorbance reading.*

Figure 3: Flow Chart of α-Synuclein ELISA Protocol.



Calculation of Results

A. MONITORING TMB SUBSTRATE REACTION

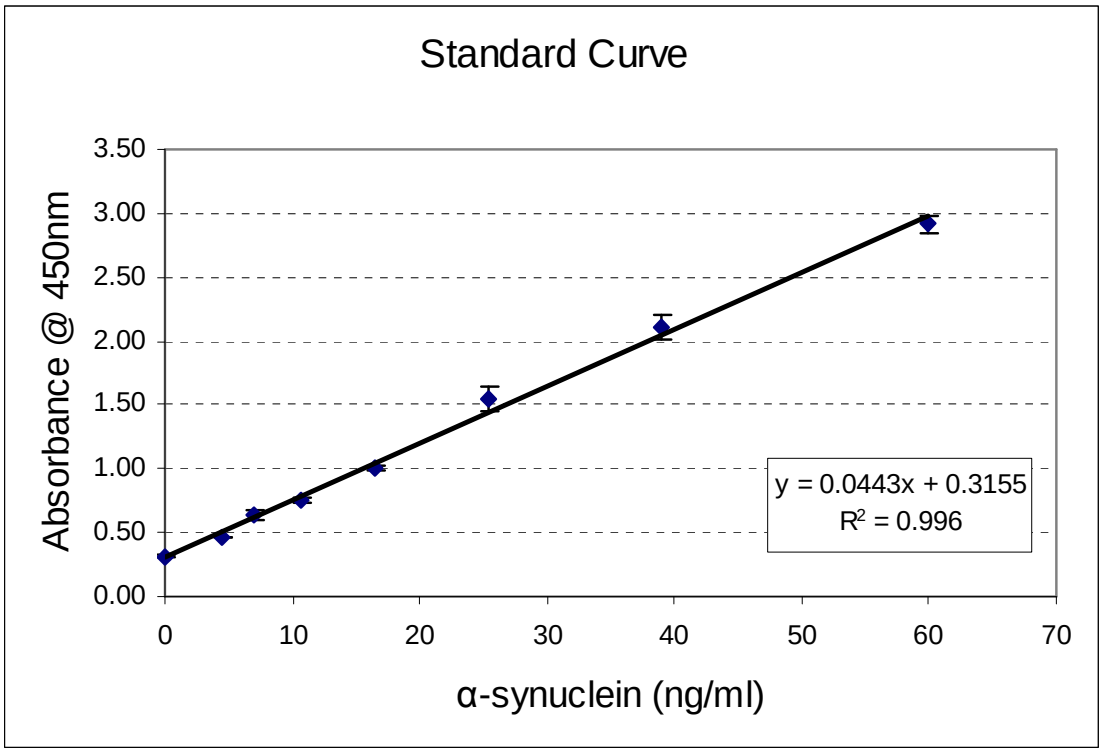
Color development should be monitored following the addition of the TMB substrate (Step 12 of the Assay Protocol). The stop solution should be added when the reaction of the highest concentration standard becomes deep blue in color. Over development should be avoided as this can lead to precipitation of the colored product or "out of range" reads. It is possible to monitor the color development using a spectrophotometer. This can be done by taking periodic reads at 620 nm until an OD of 0.6 to 0.9 is reached for the highest standard (60ng/ml) at which time the stop solution should be added.

B. STANDARD CURVE

As discussed previously, for each α-Synuclein ELISA experiment that is performed a standard curve must be included to allow subsequent quantitation of α-Synuclein in unknown samples. Listed below are example absorbance readings from a set of standards that were prepared following the protocol detailed above.

α-Synuclein standard (ng/ml)	Sample 1	Sample 2	Ave
0.0	0.323	0.314	***0.32***
4.5	0.472	0.469	***0.47***
7.0	0.612	0.667	***0.64***
10.7	0.773	0.748	***0.76***
16.5	1.013	0.998	***1.01***
25.4	1.608	1.477	***1.54***
39.0	2.175	2.032	***2.10***
60.0	2.961	2.860	***2.91***

The results obtained should be scanned briefly to verify that the duplicates are in good agreement (i.e. low standard deviations). Then the results can be plotted on a graph with the average absorbance readings on the Y-axis and the α-Synuclein protein concentration on the X-axis as shown below.



A trend line can be calculated using linear regression which produces an equation for the line (y=0.0443x + 0.3155) as well as an R-squared value (which shows how well the data fits the line). The equation generated for that assay can then be used to solve for unknown samples run concurrently to determine their α-Synuclein concentration. To determine the α-Synuclein concentration of unknown samples requires algebraic manipulation of the standard curve equation. It has to be modified to solve for "x" (protein concentration) instead of "y" (absorbance). From the above example:

y = 0.0443 x + 0.3155

becomes

x = (y – 0.3155) / 0.0443

Thus, to determine the α-Synuclein concentrations (ng/ml) simply enter the absorbance measurement (i.e. "y") into the equation and calculate. In this example if an unknown sample yields an average absorbance reading (OD_{450}) of 1.87 then the protein concentration calculation is as follows:

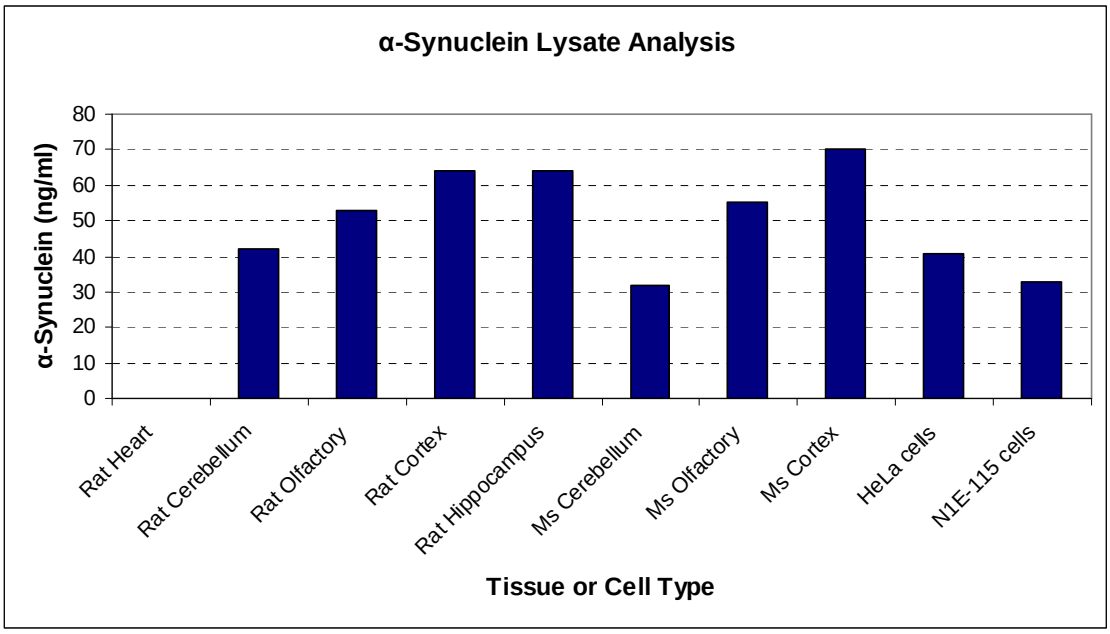
= (absorbance – 0.3155) / 0.0443
= (1.87 – 0.3155) / 0.0443
= 35.1 ng/ml

Therefore the amount of α-Synuclein protein in this sample would be 35.1 ng/ml.

C. EXAMPLE DATA

An experiment was carried out to demonstrate the effectiveness of the α-Synuclein ELISA assay. Briefly, different cells types were plated in a tissue culture dish. Protein lysates of the cultures were prepared as described above in the "Preparation of samples" section. Brain tissue was also isolated from adult mice or rats and protein lysates were prepared as described above. A Bradford assay was

conducted to determine protein concentrations of all samples. The lysates were diluted to 100 µg/ml using ELISA Diluent and then tested in the α-Synuclein ELISA assay (concurrently with protein standards). A standard curve was generated and the concentration of α-Synuclein protein in each lysate was determined (as described in section B above). The quantity of α-Synuclein protein in each sample was as follows:



References

1. Characterization of a novel protein regulated during the critical period for song learning in the zebra finch. George JM, Jin H, Woods WS, Clayton DF. Neuron. 1995; 15:361–372.
2. Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. Abeliovich, A, Schmitz Y, Farinas I, Choi-Lundberg D, Ho WH, Castillo PE, Shinsky N, Verdugo JM, Armanini M, Ryan A et al. Neuron. 2000; 25: 239–252.
3. Synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons. Murphy DD, Rueter SM, Trojanowski JQ, Lee VM. J Neurosci. 2000; 20:3214–3220.
4. Regulation of phospholipase D2: selective inhibition of mammalian phospholipase D isoenzymes by alpha- and beta-synucleins. Jenco JM, Rawlingson A, Daniels B, Morris AJ. Biochemistry. 1998; 37:4901–4909.
5. Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. Colley WC, Sung TC, Roll R, Jenco J, Hammond SM, Altshuller Y, Bar-Sagi D, Morris AJ, Frohman MA. Curr Biol. 1997; 7:191–201.
6. Update on the pathogenesis of Parkinson's disease. Schulz JB. J Neurol. 2008 Sep; 255 Suppl 5:3-7.
7. Metal-dependent generation of reactive oxygen species from amyloid proteins implicated in neurodegenerative disease. Allsop D, Mayes J, Moore S, Masad A, Tabner BJ. Biochem Soc Trans. 2008 Dec; 36(Pt 6):1293-8.
8. Multiple system atrophy: alpha-synuclein and neuronal degeneration. Yoshida M. Neuropathology. 2007 Oct; 27(5):484-93.

Warranty

Millipore Corporation (“Millipore”) warrants its products will meet their applicable published specifications when used in accordance with their applicable instructions for a period of one year from shipment of the products. **MILLIPORE MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.** The warranty provided herein and the data, specifications and descriptions of Millipore products appearing in Millipore’s published catalogues and product literature may not be altered except by express written agreement signed by an officer of Millipore. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and if given, should not be relied upon.

In the event of a breach of the foregoing warranty, Millipore’s sole obligation shall be to repair or replace, at its option, the applicable product or part thereof, provided the customer notifies Millipore promptly of any such breach. If after exercising reasonable efforts, Millipore is unable to repair or replace the product or part, then Millipore shall refund to the Company all monies paid for such applicable Product. **MILLIPORE SHALL NOT BE LIABLE FOR CONSEQUENTIAL, INCIDENTAL, SPECIAL OR ANY OTHER DAMAGES RESULTING FROM ECONOMIC LOSS OR PROPERTY DAMAGE SUSTAINED BY ANY COMPANY CUSTOMER FROM THE USE OF ITS PRODUCTS.**

Unless otherwise stated in our catalog or other company documentation accompanying the product(s), our products are intended for research use only and are not to be used for any other purpose, which includes but is not limited to, unauthorized commercial uses, in vitro diagnostic uses, ex vivo or in vivo therapeutic uses or any type of consumption or application to humans or animals.