biosensis

Make sense of your research!

biosensis® α-Synuclein *Rapid*™ ELISA Kit: Human, Mouse, Rat

Catalogue Number: BEK-2216-1P/2P

For the quantitative determination of α-synuclein in human citrate plasma, serum, cerebrospinal fluid (CSF), and mouse and rat brain homogenates only if used as directed.

Please refer to the Sample Preparation Section for specific use instructions for substrate application

For research use only, not for use in clinical and diagnostic procedures.

Table of Contents

1. Intended Use 2
2. Introduction 2
3. Materials Provided and Storage Conditions 3
4. Equipment Required but Not Supplied 3
5. Before You Start…. 3
6. Sample Preparation 3
7. Preparation of α-synuclein Standard 5
8. Other Reagents and Buffer Preparation 5
9. Assay Procedure 5
10. Technical Hints 6
11. Calculation of Results 7
12. Typical Data 7
13. Informational References 13
14. Other Information 13

Appendix A: Determining the Number of Microplate Wells Needed for ELISA Experiments 14
Appendix B: Tissue Homogenate Preparation Procedure (RIPA Buffer) 16
Appendix C: Tissue Homogenate Preparation Procedure TBS Buffer 17
Appendix D: Troubleshooting Guide 18

1. Intended Use

The purpose of this kit is the quantitative determination of human α-synuclein in human citrate plasma, human serum, human CSF, and mouse/rat brain homogenate samples only if used as directed. This kit has not been tested for other sample applications. Non-validated samples may contain immunoglobulins and other components that potentially interfere with this ELISA assay. Biosensis does not assume responsibility if this kit is used for unintended purposes.

For research use only. Not for diagnostic and clinical purposes.

2. Introduction

Alpha-synuclein is a member of the synuclein family of proteins, which also includes β-synuclein and γ-synuclein (1). α-synuclein is an abundant 140 amino acid protein, expressed primarily at presynaptic terminals in the central nervous system (1). In healthy cells, α-synuclein exists mainly in it's monomeric form, however, in diseased cells, aggregation leads to soluble oligomeric and insoluble fibril forms of the protein (2). α-synuclein had been described extensively in the literature, including in brain tissue, cerebrospinal fluid (CSF), in serum, plasma, saliva, megakaryocytes in bone marrow, and in the erythrocytes and platelets in blood (3-8). Interestlingly, measurements of α-synuclein vary between source and also between reports. Stefaniuk *et al* 2018, found that α-synuclein increased in single donor platelets (SDP) plasma in storage over time (5). Ishil *et al* (2015) noted that once heterophilic interference is eliminated, α-synuclein levels in patients with PD were significantly lower than controls (4). While the levels of α-synuclein vary amoung tissues and source, it is clear from the literature, that α-synuclein, or α-synucleinopathy, plays a major part in Parkinson's Disease (PD), dementia and the formation of Lewy bodies (DLB), and therefore maybe a useful biomarker in these diseases (9).

The Biosensis α-Synuclein *Rapid*™ enzyme-linked immunosorbent assay (ELISA) Kit is a sandwich ELISA that allows the preferential quantification of α-synuclein in less than 4 hours. This kit consists of a pre-coated anti-α-synuclein capture antibody (amino acids: 116-131), a biotinylated anti-α-synculein synuclein detection antibody (region 61-95) and horseradish peroxidase (HRP)-conjugated streptavidin. The addition of a substrate (3,3',5,5'-tetramethylbenzidine, TMB) yields a coloured reaction product which is directly proportional to the concentration of α-synuclein present in samples and protein standards.

The α-synuclein ELISA kit employs a recombinant human standard expressed in *E.coli*. The provided Quality Control (QC) sample serves as a positive control with a defined concentration range of α-synuclein protein, formulated in a stabilized buffer solution and designed to assure consistent assay performance. The high degree of amino acid homology of α-synuclein among species allows quantification of α-synuclein in mouse and rat samples, and results may be expressed as "human α-synuclein equivalents".

The purpose of this kit is for the in vitro measurement of α-synuclein in human citrate plasma, serum, cerebrospinal fluid (CSF), and mouse and rat brain homogenates. This kit has not been tested for other applications.

Sufficient amount of α-synuclein standard is supplied to allow for spike-and recovery experiments in order to validate this ELISA assay for other sample matrices if required. For further assistance with assay validation please refer to Technical Note #1 on the Biosensis website.

3. Materials Provided and Storage Conditions

Reagent	Quantity	
	1 Plate Kit	2 Plate Kit
α-synuclein antibody coated 96-well microplate	12 x 8 wells	24 x 8 wells
Sample Diluent A (1x)	1 x 33 mL	2 x 33 mL
Assay diluent A (1x)	1 x 25 mL	2 x 25 mL
Recombinant human α-synuclein standard	2 x 20 ng	4 x 20 ng
Quality Control (QC) Sample	2 Vials (see vial label for amount)	4 Vials (see vial label for amount)
α–synuclein detection antibody (100x)	1 x 110 µL	2 x 110 µL
Streptavidin-HRP (100x)	1 x 110 µL	2 x 110 µL
Wash buffer (10x)	1 x 33 mL	2 x 33 mL
TMB substrate (1x)	1 x 11 mL	2 x 11 mL
TMB stop solution (1x)	1 x 11 mL	2 x 11 mL
Plate sealer	Supplied	

Reagent	Storage and Stability
ELISA kit as supplied & unopened	12 months at 2-8°C
Reconstituted standard and QC sample	Do not store, use on same day
Diluted detection antibody and HRP conjugate (1x)	2 weeks at 2-8°C
Diluted wash buffer (1x)	2 weeks at 2-8°C

Note:

- Do not freeze the streptavidin-HRP conjugate
- Do not use kit components from other ELISA kits

4. Equipment Required but Not Supplied

- Single- and multi-channel pipettes capable of delivering 10-1000 µL volumes
- Plastic and glass ware for sample collection, sample preparation and buffer preparation
- Plate shaker
- Microplate reader capable of reading absorbance at 450 nm

5. Before You Start….

- Read the entire protocol to familiarize yourself with the assay procedure; a tutorial about the use of *Rapid*™ ELISA kits is available online
- Bring the microplate and all reagents and solutions to room temperature before starting the assay
- Remove only the required amount of TMB substrate from the fridge to prevent multiple warm-ups and cool downs; keep the TMB substrate in the dark
- Remove the number of strips required and return unused strips to the pack and reseal
- Centrifuge all kit components to collect reagents to the bottom of tubes before use
- The stop solution provided with this kit is an acid; wear protective equipment when handling
- Please visit our Technical Notes section at www.biosensis.com for further information and helpful hints on ELISA-related topics

Careful experimental planning is required to determine the total number of samples that can be assayed per plate. Refer to Appendix A for help in determining the number of microplates required to conduct an ELISA assay experiment.

6. Sample Preparation

This kit contains **Assay Diluent A** and **Sample Diluent A**. Assay Diluent A is used to dilute the detection antibody and streptavidin-HRP conjugate. Sample Diluent A is used to dilute samples only, and is optimized for measuring α-synuclein in human citrate plasma, serum, CSF, and mouse/rat brain extracts. See the following sample preparation guidelines for specific substrate preparation recommendations and sample dilution instructions.

For unknown concentrations of α-synuclein in samples, it is important to perform several dilutions of the sample to allow the α-synuclein concentration to fall within the range of the α-synuclein standard curve (0.16-10 ng/mL).

Also, with unknown samples of all types it is highly recommended to run parallelism/dilutional linearity

experiments and α-synuclein spike- and recovery control tests over a short range of dilutions using our standard to help evaluate the particular sample performance in the assay. Spike-recovery experiments that follow a reasonably linear progression and achieve a spiked recovery of 80-120% of spiked value demonstrates that the subject samples are performing acceptably in the assay. Failure of spiked recovery samples indicates that sample buffer, preparation and dilution and or blocking procedures will need to be optimized by the end user for consistent, accurate results with this ELISA assay. For further assistance please refer to Technical Note #1 on www.biosensis.com.

Human Citrate-Plasma

The detection of α-synuclein in human plasma has been widely reported in the literature, however levels vary considerably from pg/mL to high ng/mL depending on plasma preparation and detection method. Furthermore, it has been shown that α-synuclein can increase steadily over time with storage. (5). **Thus, to prevent sample variation, strict adherence to consistent sample preparation procedures among samples and study groups are required.**

Internal testing suggests the following collection and preparation guidelines for human citrate plasma. We however strongly recommend reviewing relevant literature for assaying α-synuclein in human blood. The following procedure is therefore only a guideline.

- Collect plasma using citrate as anticoagulant; EDTA-treated and heparin-treated plasma samples have not been tested as yet
- Centrifuge for 15 min at 2-8°C at 1,500 x g within 30 minutes of collection
- For eliminating the platelet effect we suggest further centrifugation for 10 min at 2-8°C at 10,000 x g
- It is recommend to analyse samples immediately or aliquot and store samples at -80°C, thawing only once.
- The minimum required dilution (MRD) for human citrate plasma samples is generally 1/10, but this varies between samples. Human citrate plasma samples with low levels of α-synuclein can be diluted 1/2, however for samples that show high interference, a higher dilution is recommended for better accuracy. The kit contains enough standard to perform parallelism/dilutional linearity experiments and α-synuclein spike- and recovery if required.
- Dilute human citrate plasma samples with **Sample Diluent A** in order to measure α-synuclein concentrations.

Human Serum

Important: To prevent hemolysis of red blood cells and to ensure reproducibility of results careful procedures must be adhered to when collecting, processing and storing blood samples, because α-synuclein is stored in red blood cells (10).

- Blood samples should be collected in a separate tube with separator gel (10) and then centrifuged immediately
- Centrifuge at approximately 1,000 x g at 2-8°C for 15 minutes
- Analyze the serum immediately or aliquot and store frozen in aliquots at -80°C; Do note store at lower temperatures.
- For human serum samples, a minimum dilution of 1/5 – 1/10 with **Sample Diluent A** is required.

Optimization of dilutions for serum samples are recommended for best results.

For larger studies involving multiple assays across days, or using multiple reagent batches we recommend the use of a citrate-plasma sample and serum samples with known concentration as internal control for best assay accuracy.

Cerebrospinal Fluid (CSF)

Collect CSF samples by lumbar puncture into conical polypropylene tubes and mix gently (11). Samples should then be centrifuged at 2,000 x g for 10 minutes at 2-8°C. Test samples immediately or aliquot and freeze at -80°C within 30 minutes of collection (11).

- Dilute human CSF samples with **Sample Diluent A** in order to measure α-synuclein concentrations.
- The minimum required dilution (MRD) for human CSF samples is generally 1/2, but this can vary between samples. **Some samples may read below the limit of detection (LOD) of this kit.**

Note: High-quality CSF samples are required for best results. Contamination of CSF fluid with blood can cause assay interference of blood factors and thus inaccurate results.

Brain Tissue Extracts

Alpha-synuclein concentrations in brain extracts will vary based on the extraction method and tissue sample (see data presented in Section 12). Optimal sample dilutions with Sample Diluent A will need to be determined empirically by the end-user, as they can vary from 1:4 to 1:100. Initial testing should involve performing a serial sample dilution over a large range to determine the required sample dilutions, prior to testing all study samples.

This kit has been tested on RIPA- and TBS- extracted brain tissue homogenates. Refer to **Appendix B** and **Appendix C** for detailed sample preparation protocols.

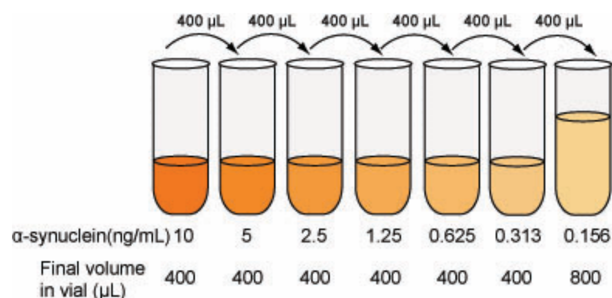
7. Preparation of α-synuclein Standard

Note: Equilibrate all reagents to room temperature for 30 minutes, and then mix all buffers thoroughly before proceeding.

- Reconstitute the lyophilized antigen standard with 1 mL of **Sample Diluent A**
- Label the vial with the reconstituted α-synuclein standard as "20 ng/mL"; vortex and let stand for 15 minutes
- Dilute the 20 ng/mL α-synuclein standard 1:2 (eg., 400 µL of 20 ng/mL standard + 400 µL diluent); label this tube "10 ng/mL"

In order to generate an α-synuclein standard curve, perform a 1:2 serial dilution down to 0.16 ng/mL. The volumes used for the dilution series depends on the number of repeats per α-synuclein concentration. For triplicate measurement (100 µL per well) of each α-synuclein standard concentration, we recommend this procedure:

1. Label 6 tubes with "5.0 ng/mL", "2.5 ng/mL", "1.25 ng/mL", "0.625 ng/mL", "0.313 ng/mL" and "0.16 ng/mL", respectively
2. Aliquot 400 µL of the sample diluent into each tube
3. Take 400 µL from the "10 ng/mL" tube and transfer to the "5.0 ng/mL" tube
4. Pipet up- and down and vortex to mix; to avoid foaming, use only a very brief vortex
5. Repeat steps 3 and 4 for each consecutive concentration until the last tube "0.16 ng/mL" is prepared and mixed well



8. Other Reagents and Buffer Preparation

- Quality Control (QC) sample: Reconstitute the lyophilized sample with 1 mL of **Sample Diluent A**. This will provide a QC sample within 1.75 – 3.25 ng/mL.
- Detection antibody (100x) and streptavidin-HRP conjugate (100x): dilute one hundred-fold with **Assay Diluent A;** do not use Sample Diluent A. Prepare enough volume to add 100 µL per well of both detection antibody and streptavidin-HRP.
- Wash buffer (10x): crystals may form at the bottom of the bottle; equilibrate to room temperature, mix thoroughly and dilute ten-fold with ultrapure water; each 8-well strip requires about 25 mL of 1x wash buffer per assay

9. Assay Procedure

All steps are performed at room temperature (20-25°C, 70-75°F).

1. Add 100 µL of diluted α-synuclein standards, QC sample, samples and blank (sample diluent only) to the pre-coated microplate wells
2. If available, include sample-specific negative and positive control sample in the assay procedure
3. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 120 minutes

4. Discard the solution inside the wells and perform 5 washes with 1x wash buffer (200 μL per well). See the technical hints section for a detailed description of the washing procedure
5. Add 100 μL of the detection antibody (1x) into each well
6. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 60 minutes
7. Discard the solution inside the wells and wash as described in step 4
8. Add 100 μL of the 1x streptavidin-HRP conjugate into each well
9. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 30 minutes
10. Discard the solution inside the wells and wash as described in step 4
11. Add 100 μL of TMB into each well and incubate plate at room temperature for 15-30 minutes without shaking in the dark
12. Stop the reaction by adding 100 μL of the stop solution into each well. Visible blue color will change to yellow. Read the absorbance at 450 nm on a plate reader. **Note:** Color will fade over time; hence, we recommend plate to be read within 5 minutes after adding the stop solution or no longer than 30 minutes after addition
13. See Appendix D for a troubleshooting guide when unexpected difficulties are encountered

* RCF= 1.12 x Orbit Radius x $(rpm/1000)^2$

10. Technical Hints

1. Do not perform dilutions within the well
2. At least duplicate measurements for each standard and sample dilution is recommended
3. Dilute samples to an α-synuclein concentration that falls within the range of the standard curve. Do not extrapolate absorbance readings
4. Avoid touching the inside surface of the wells with the pipette tip
5. Proper emptying and washing the plate is crucial for low background and non-specific binding. For manual plate washing, we recommend the following procedure:
   a. To remove liquid from the wells, place the plate on the palm of the hand and quickly invert the plate over the sink. Forcefully move the arm downwards and stop abruptly to force the liquid out of the wells. When done correctly, this technique should prevent liquid from getting onto the fingers or onto the outside of the microplate wells
   b. Blot and forcefully tap the microplate against clean paper towels for 3-5 times
   c. Wash the wells by pipetting 200 μL of wash buffer into each well and empty the wells as described in step a-b)
   d. Repeat this procedure for a total of 5 times
6. Complete removal of liquid from the wells at each step is essential for reliable results. However, avoid letting the wells dry out at any time
7. Add TMB and the stop solution to the wells in the same order
8. Ensure that all bubbles are removed and that the bottom of the plate is optically clean prior to taking the absorbance reading
9. Stopping the TMB reaction after 15-30 minutes is usually sufficient to obtain a standard curve. However, TMB incubation times may vary based on the accuracy of diluting the ELISA kit reagents and differences in incubation time and temperature. We use a plate shaker set to 140 rpm (0.351 G) and perform all incubations at room temperature (20-25˚C, 70-75˚F) in our laboratories. Importantly, we read our plates on a microplate reader capable of measuring up to an optical density (OD) of 4. You may need to adjust the TMB incubation time if your microplate reader has a more limited range.
10. TMB incubation times can vary, and assay sensitivity can be affected if color development is stopped too early. In order to determine the optimal time to stop the TMB incubation, blue color development should be monitored at **650 nm**. Addition of Stop Solution will convert blue into yellow color, with an ~2.5-3X increase in OD at **450 nm**. Once the highest standard reaches an OD of ~1.0 at 650 nm, immediately stop the reaction by adding Stop Solution. Note that before taking a 650 nm measurement, the plate should be briefly and gently shaken by hand to homogenise the blue TMB

reaction product within the wells for most accurate readings.

11. Calculation of Results

1. Average the readings for each α-synuclein standard concentration, blank and sample
2. Plot a standard curve with the α-synuclein standard concentration on the x-axis and the OD at 450 nm on the y-axis
3. If values for the α-synuclein standards are adjusted for background absorbance, then subtract the blank value from the OD_{450} of the samples as well
4. Use appropriate software to reduce the data and generate a four parameter logistic (4-PL) curve-fit; avoid using linear regression analysis
5. Perform a 4-PL regression analysis to calculate the concentration of α-synuclein in the QC sample. An observed concentration within the range of 1.75 – 3.25 ng/mL when using the extended standard curve range, see Section 7) indicates acceptable assay performance
6. Perform a 4-PL regression regression analysis to calculate the concentration of α-synuclein in the samples. Multiply the result by the sample dilution factor

Manual Plate Reading

The relative OD_{450} = (the OD_{450} of each well) – (the OD_{450} of Zero well and/or Blank well).

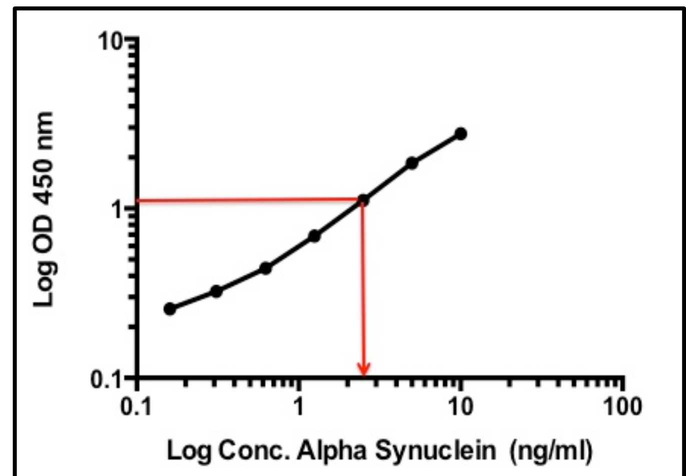
1. The **standard curve** can be plotted as the relative OD_{450} of each α-synuclein standard solution (Y-axis) vs. the respective known concentration of the α-synuclein standard solution (X-axis). The standard curve should result in a graph that shows a direct relationship between target protein concentrations and the corresponding OD_{450}. The greater the concentration of target protein in the solution, the higher the OD_{450}.

2. **Determine concentration of target protein in unknown sample:** The α-synuclein protein concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of α-synuclein in the unknown sample. In the exemplary standard curve below, a sample with OD_{450} = 1.12 reads as 2.5 ng/mL α-synuclein (red line). If the samples measured were diluted, multiply the concentrations from the interpolation with the dilution factor to obtain the actual α-synuclein concentration in the sample.

12. Typical Data

Standard Curve

Standard curves are provided for demonstration only. A standard curve has to be generated for each α-synuclein ELISA assay.



In the example Figure above and Table below α-synuclein standards were run in triplicates. The TMB reaction was stopped after 20 minutes.

Typical optical densities and coefficient of variations for diluted α-synuclein standards are summarized in the following Table:

α-synuclein [ng/mL]	Mean	CV (%)
10.00	2.76	2
5.00	1.85	3
2.50	1.12	4
1.25	0.69	2
0.63	0.44	2
0.31	0.32	4
0.16	0.26	5
Blank	0.20	5

CV: Coefficient of Variation

Limit of Detection

This α-synuclein ELISA kit detects a minimum of 100 pg/mL α-synuclein in sample buffer (defined as concentration at Blank OD450 plus 3x standard deviation of blank OD, n=10).

Specificity

This ELISA kit detects α-synuclein and has ≤17% cross-reactivity with the oligomeric form. Other α-synuclein forms have not been tested however, it is not expected to discriminate between various α-synuclein isoforms.

Interference and cross-reactivity of human β- and γ-synuclein was assessed by spiking each protein at excess concentration of 20 ng/mL into two human plasma samples, each diluted 1:10 and 1:20, in comparison to unspiked samples. Cross-reactivity was calculated based on increase or decrease of apparent α-synuclein concentrations in spiked samples.

Sample	Cross-Reactivity of Spiked Protein	
	β-synuclein	γ-synuclein
Plasma 1	0.9 %	2.6 %
Plasma 2	1.5 %	2.0 %
Average	**1.2 %**	**2.3 %**

At a 3-6 fold excess (w/v) of spiked protein over endogenous α-synuclein at both sample dilutions, this ELISA shows very little cross-reactivity of 2.3% or less.

Species Cross-Reactivity

This kit has only been tested on human, mouse and rat samples and species cross-reactivity has been observed. Other species have not been tested.

Intra- and Inter-Assay Coefficient of Variation (CV)

To determine the reproducibility of the α-synuclein ELISA, six human citrate plasma samples were tested by three different operators.

Sampes were diluted in Sample Diluent A and measured in triplicate wells. Sampes were tested three times across one 96-well plate for a total of 9 replicate wells per plate per operator per sample. Samples and sample dilutions were chosen according to the minimal required dilution (MRD) for each sample, covering the entire range of the calibration curve.

The **intra-assay CV** was calculated for 3 sample repeats (each assayed in triplicate wells) for each of the 6 plasma samples on each ELISA plate for 3 operators. The intra-assay CV ranged from 5.1 – 14.5 % (average: 8.2 %).

INTRA-ASSAY Coefficient of Variation							
Sample		1	2	3	4	5	6
Op. 1	n	3	3	3	3	3	3
	Mean (ng/mL)	66.9	20.6	9.9	9.5	2.8	33.1
	Stdev (ng/mL)	5.0	1.2	0.7	1.2	0.4	1.9
	CV (%)	**7.5**	**5.8**	**7.1**	**12.6**	**14.3**	**5.7**
Op. 2	n	3	3	3	3	3	3
	Mean (ng/mL)	70.2	19.8	8.6	8.6	2.6	35.6
	Stdev (ng/mL)	5.5	1.0	0.5	1.0	0.2	2.3
	CV (%)	**7.8**	**5.1**	**5.8**	**11.6**	**7.7**	**6.5**
Op. 3	n	3	3	3	3	3	3
	Mean (ng/mL)	71.0	18.9	8.1	8.3	2.3	35.7
	Stdev (ng/mL)	4.8	1.1	0.5	1.2	0.2	2.7
	CV (%)	**6.8**	**5.8**	**6.2**	**14.5**	**8.8**	**7.6**

n: number of replicate wells; Stdev: Standard Deviation; CV: Coefficient of Variation; Op: Operator.

The **inter-assay CV** was calculated by taking into account sample concentrations obtained for 6 samples across 3 plates, each plate assayed by 1 operator. The inter-assay CV ranged from 6.1 – 13.6 % (average: 9.4 %), demonstrating excellent reproducibility.

INTER-ASSAY Coefficient of Variation						
Sample	1	2	3	4	5	6
n	9	9	9	9	9	9
Mean	69.3	19.8	8.9	8.8	2.6	34.8
Stdev	4.8	1.2	1.0	1.2	0.3	2.4
CV (%)	**6.9**	**6.1**	**11.2**	**13.6**	**11.5**	**6.9**

n: number of replicate wells; Stdev: Standard Deviation; CV: Coefficient of Variation

Quantification of α-synuclein in Human Citrate Plasma

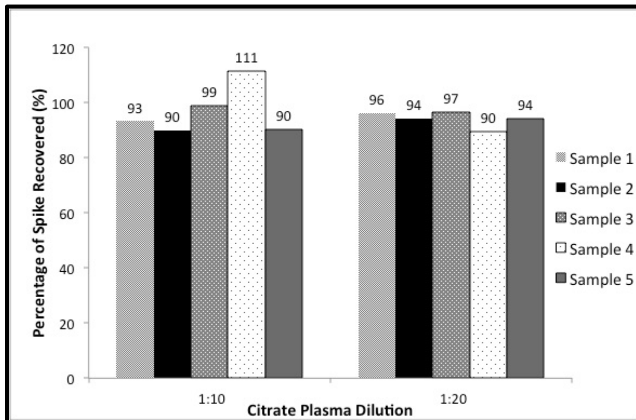
Note: All blood samples assayed have been obtained from healthy individuals. Patient samples have not been tested as yet.

Five human citrate plasma samples were diluted 1/10 and 1/20 in Sample Diluent A and assayed in duplicate on two separate days by 1 operator. The concentration of α-synuclein in human citrate plasma ranged from 3.2 to 40.9 ng/mL as shown in the following table.

Plasma (Citrate)	Dilution	Assay Repeat		Mean (ng/mL)
		1	2	
1	1:10	2.6	3.7	**3.2**
	1:20	2.4	4.0	
2	1:10	11.9	12.7	**11.9**
	1:20	11.7	11.4	
3	1:10	39.1	40.8	**40.9**
	1:20	40.4	43.4	
4	1:10	5.5	4.6	**5.3**
	1:20	6.1	4.9	
5	1:10	4.8	5.9	**5.4**
	1:20	4.6	6.2	

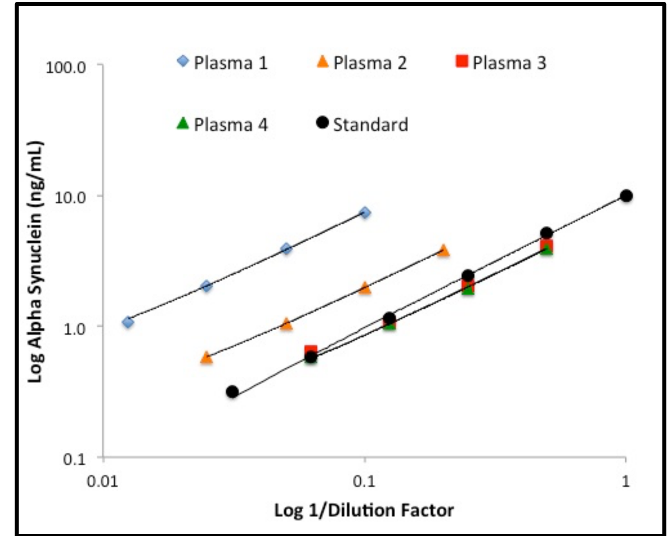
Recovery of α-synuclein in Human Citrate Plasma

The same five human citrate plasma samples above were spiked with 2.5 ng/mL α-synuclein protein and diluted 1/10 and 1/20 in Sample Diluent A. The recoveries (90-111%) were all in an acceptable range of 80-120%.



Linearity-of-Dilution and Parallelism (Citrate-Plasma)

Four human citrate plasma samples were diluted across a range of dilutions in Sample Diluent A and assayed in triplicate wells. Parallelism of diluted samples to the reference standard curve was assessed for each sample and plotted in the graph below.



Parallelism was also assessed by calculating the α-synuclein concentration at each dilution as the percent recovery of previous dilution (target: 80-120%), and the inter-dilution CV (target: ≤ 20%).

Plasma	OD 450	DF	ng/mL (corr)	Inter-Dilution CV (%)	Mean ng/mL	% of prev. dil.
1	2.292	10	74.3	**6.5**	**80.6**	100
	1.535	20	79.0			106
	0.974	40	82.3			104
	0.628	80	86.8			105
2	1.505	5	19.2	**8.7**	**20.9**	100
	0.944	10	19.7			103
	0.620	20	21.3			108
	0.431	40	23.2			109
3	1.583	2	8.27	**10.9**	**8.82**	100
	0.962	4	8.09			98
	0.630	8	8.72			108
	0.454	16	10.21			117
4	1.536	2	7.91	**8.1**	**8.40**	100
	0.943	4	7.87			99
	0.618	8	8.47			108
	0.432	16	9.33			110

DF: Dilution Factor; corr: corrected for dilution factor; CV: coefficient of variation

The values for all four plasma samples displayed excellent parallelism, therefore demonstrating accurate quantification of α-synuclein in human citrate-plasma. The data also shows that the optimal dilution range and MRD is sample-specific, and can vary between 1:2 to 1:10.

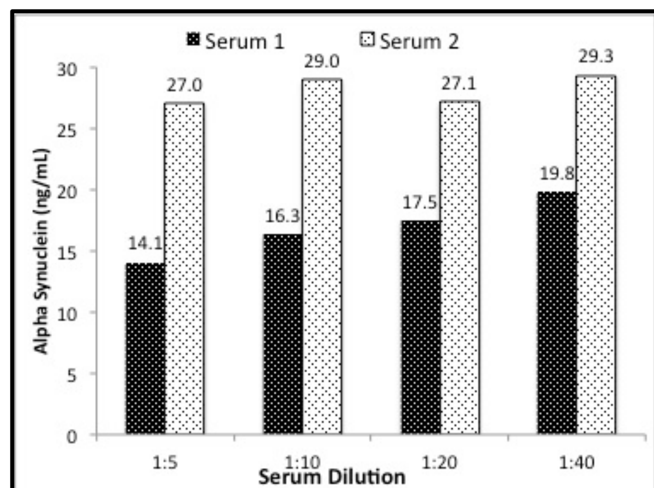
Spike-Recovery of α-synuclein in Human Serum

Two human serum samples were diluted at 1:5 to 1:40 in Sample Diluent A, with and without a spike of α-synuclein (2.5 ng/mL) protein, and then tested in duplicate.

The mean percentage of spike recovered across the four dilutions was 100 and 112% respectively for serum 1 and serum 2.

Percentage of α-synuclein spike recovered (%)					
Serum	**Dilution**				**Mean**
	1:5	1:10	1:20	1:40	
1	102	108	96	94	100
2	136	112	104	94	112

The mean concentrations of α-synuclein measured in human serum above were 17 and 28 ng/mL, respectively, for serum 1 and serum 2.



Parallelism and Inter-Assay CV in Human Serum

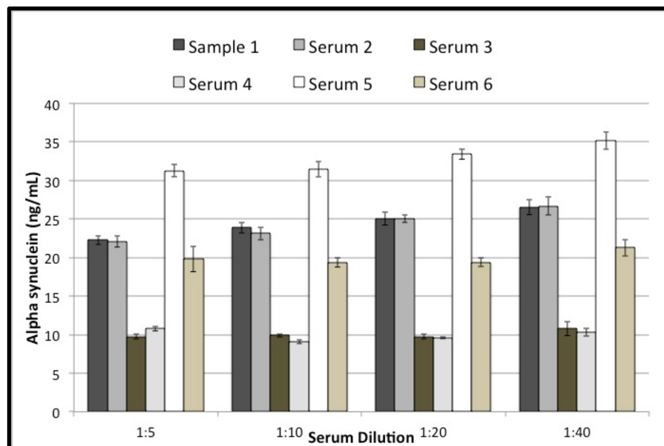
Six human serum samples were diluted 1/5 to 1/40 in Sample Diluent A and assayed in triplicate wells at each dilution on four separate days by one operator.

The following table contains dilution-corrected concentrations for α-synuclein for each sample at all four dilutions. Parallelism was assessed by calculating the % recovery of α-synuclein in relation to the concentration at previous dilution (target: 80-120%) and the inter-dilution CV (target: ≤ 20%). Excellent parallelism and thus accurate quantification in human serum was achieved.

	DF	**Conc. (DF corrected) in 4 Assays / [ng/mL]**				**Mean ng/mL**	**Inter-dilution CV (%)**	**% of prev dil.**
		#1	**#2**	**#3**	**#4**			
1	5	22.1	21.6	21.4	24.0	22.3	7.3	100
	10	23.5	24.1	22.3	25.6	23.9		107
	20	24.2	25.1	23.6	27.4	25.1		105
	40	24.8	28.2	24.8	28.3	26.5		106
2	5	23.0	20.5	21.2	23.7	22.1	8.4	100
	10	23.6	21.3	22.3	25.2	23.1		105
	20	26.1	24.4	24.1	25.5	25.0		108
	40	24.6	29.4	24.9	27.6	26.7		107
3	5	9.3	9.8	9.2	10.6	9.7	5.0	100
	10	9.6	10.4	9.5	10.1	9.9		102
	20	9.7	10.0	9.0	10.4	9.8		99
	40	11.2	13.2	9.7	9.1	10.8		111
4	5	9.5	8.3	8.8	9.7	9.1	6.0	100
	10	9.5	9.5	9.2	10.2	9.6		106
	20	10.5	10.3	9.9	10.4	10.3		107
	40	10.2	11.7	9.7	9.4	10.3		100
5	5	31.6	30.6	33.3	29.5	31.3	5.7	100
	10	33.3	31.7	28.5	32.1	31.4		101
	20	34.5	32.1	34.7	32.4	33.4		106
	40	36.2	37.8	32.5	34.2	35.2		105
6	5	18.9	17.8	24.6	17.8	19.8	4.6	100
	10	19.1	19.1	21.0	18.2	19.3		98
	20	20.2	20.5	18.4	18.4	19.4		100
	40	23.3	23.0	19.7	19.1	21.3		110

DF: Dilution Factor; CV: coefficient of variation

For a graphical presentation of accurate quantification, dilution-corrected concentrations for each serum sample and dilution were plotted:



The mean concentration of α-synuclein in the six human serum samples ranged from 9.8 ng/mL to 32.8 ng/mL. The inter-assay CV was calculated taking into account the mean concentration for each sample on each plate (n=4, one operator). Excellent inter-assay precision in human serum was achieved with CV ≤ 10 %.

INTER-ASSAY Coefficient of Variation						
Serum	**1**	**2**	**3**	**4**	**5**	**6**
Mean	24.4	24.2	10.0	9.8	32.8	19.9
Std Dev	2.2	2.4	0.8	0.9	2.3	2.01
CV (%)	**9.0**	**9.9**	**8.0**	**9.2**	**7.0**	**10.1**

Stdev: Standard Deviation; CV: Coefficient of Variatio

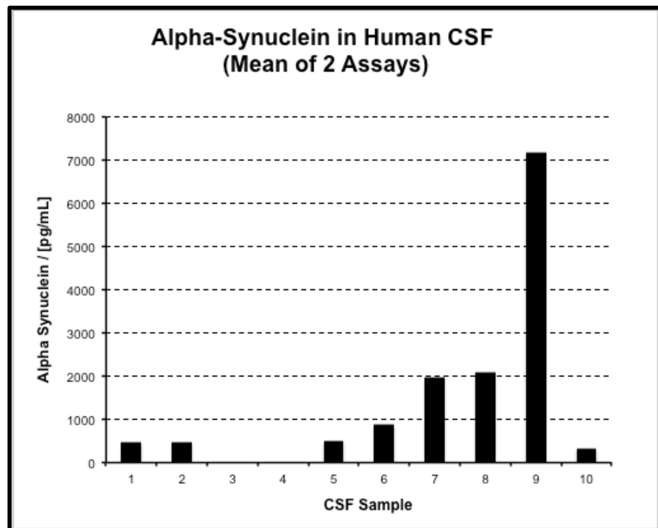
Quantification of α-synuclein in Human CSF

Ten human CSF samples were diluted 1/2 to 1/8 in Sample Diluent A and measured in duplicate wells at each dilution. These samples were tested on two separate days.

	DF	**Assay 1 (Dilution corrected)** pg/mL	Mean (Inter-Dil. CV)	**Assay 2 (Dilution corrected)** pg/mL	Mean (Inter-Dil. CV)	**Mean pg/mL**
1	2	464	464 (N/A)	480	480 (N/A)	**472**
	4	NQ		< LOD		
	8	< LOD		< LOD		
2	2	470	470 (N/A)	484	484 (N/A)	**477**
	4	NQ		< LOD		
	8	< LOD		< LOD		
3	2	NQ	NQ (N/A)	NQ	NQ (N/A)	**NQ**
	4	< LOD		< LOD		
	8	< LOD		< LOD		
4	2	< LOD	< LOD (N/A)	< LOD	< LOD (N/A)	**< LOD**
	4	< LOD		< LOD		
	8	< LOD		< LOD		
5	2	534	534 (N/A)	466	466 (N/A)	**500**
	4	NQ		NQ		
	8	NQ		< LOD		
6	2	912	908 (N/A)	892	860 (N/A)	**884**
	4	904		828		
	8	NQ		NQ		
7	2	1826	1981 (9.0 %)	1794	1998 (10.0 %)	**1989**
	4	1940		2008		
	8	2176		2192		
8	2	2130	2105 (6.4 %)	2246	2101 (7.2 %)	**2103**
	4	2224		2112		
	8	1960		1944		
9	2	7380	7328 (2.0 %)	6958	7022 (1.7 %)	**7175**
	4	7444		7156		
	8	7160		6952		
10	2	336	336 (N/A)	336	336 (N/A)	**336**
	4	< LOD		< LOD		
	8	< LOD		< LOD		

DF: Dilution Factor; LOD: Limit of Detection; NQ: Not quantifiable (concentration lower than lowest standard)

α-synuclein concentrations varied from undetectable (< LOD) to 7.2 ng/mL. Dilutional linearity was observed for samples which allowed assaying at minimum of 3 dilutions, demonstrating accurate quantification in human CSF.



Quantification of α-synuclein in Rat Brain Extracts Using Two Different Extraction Buffers

One whole rat brain (960 mg) was homogenized on a bead homogenizer with 0.96 mL of TBS buffer. A second whole rat brain (1130 mg) was homogenized in the same way with 1.130 mL of RIPA buffer. Samples were then incubated on ice for 30 min and then centrifuged at 10,000 x g for 30 min. The total protein concentration was measured by Bradford assay and aliquots were stored at -80ºC.

The brain samples were assayed for α-synuclein concentration at dilutions of 1:40 to 1:320 in Sample Diluent A, and the ELISA was repeated on 2 separate occasions by the same operator (n=2). The following table summarizes the alpha-synuclein values obtained from both the TBS and RIPA extracted rat brains.

Buffer	Mean [ng/mL]	Protein* (mg/mL)	ng α-synuclein / mg protein	Total protein (mg)	ng α-syn. / g tissue
TBS	120.8	2.222	54.4	2.1	121
RIPA	233.0	9.072	25.7	10.3	233

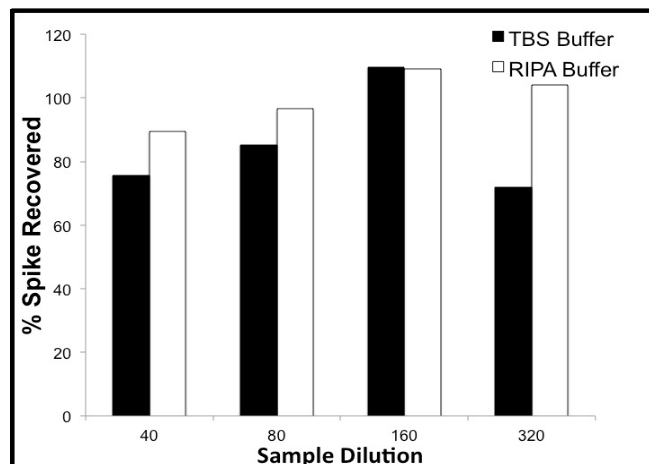
*Total protein measured by Bradford assay.

The results demonstrate that the choice of extraction buffer yields varying levels of apparent α-synuclein concentration. TBS extraction results in higher ratio of extracted α-synuclein per extracted total protein, but lower ratio of α-synuclein per tissue wet weight, as compared to RIPA-extraction. This is likely due to the nature of RIPA buffer ingredients being stronger in dissociating cellular structures and/or disrupting α-synuclein bound to binding partners. This needs to be considered when designing experimental conditions and comparing results obtained with this kit with published data.

Recovery in Rat Brain

Two rat brain samples, extracted with either TBS or RIPA buffer, were diluted 1:40 and then spiked with 2.5 ng/mL α-synuclein protein. The samples were then diluted 1:2 to total dilutions of 1:80, 1:160 and 1:320. The ELISA was repeated on 2 separate occasions (n=2) by one operator.

The recovery of spikes ranged from 76-110% in TBS buffer and 90-109% in RIPA buffer, as shown in the following figure. These results indicate that both extraction methods yield acceptable quantification in rat brain extracts, with RIPA buffer giving slightly better accuracy.



Quantification of α-Synuclein in Mouse Brain Homogenates

Mouse tissue was homogenized as follows: Cerebellum (57.5 mg), mid-brain (55 mg), cortex (100 mg) and olfactory bulb (14 mg) were homogenized on a bead homogenizer with 0.0575 mL, 0.055 mL, 0.1 mL and 0.014 mL of RIPA buffer respectively, and according to

procedure in Appendix B. In short, samples were incubated on ice for 30 min and then centrifuged at 10,000 x g for 30 min. The total protein concentration was measured by the Bradford assay and aliquots were stored at -80ºC.

To test for the concentration of alpha synuclein in the mouse brain homogenates, the samples were diluted (1:4 to 1:32 cerebellum and cortex and 1:10 to 1:80 mid-brain and olfactory) in Sample Diluent A and tested in triplicate. The following table shows the concentration of alpha-synuclein detected in each tissue sample.

Mouse Sample	Mean [ng/mL]	Protein* [mg/mL]	ng α-syn / mg protein	Total protein [mg]	ng α-syn / g tissue
Cerebellum	18.1	7.55	2.4	4.3	181
Mid-Brain	29.8	5.83	5.1	3.2	298
Cortex	58.6	7.83	7.5	7.8	586
Olfactory	38.3	3.72	10.3	0.5	383

*Total protein [mg/mL] measured by Bradford

13. Informational References

1. Fitzgerald E, Murphy S, Martinson HA. Alpha-Synuclein Pathology and the Role of the Microbiota in Parkinson's Disease. *Front Neurosci*. 2019;13:369. Published 2019 Apr 24. doi:10.3389/fnins.2019.00369
2. Lassen LB, Gregersen E, Isager AK, Betzer C, Kofoed RH, Jensen PH. ELISA method to detect α-synuclein oligomers in cell and animal models. *PLoS One*. 2018;13(4):e0196056. Published 2018 Apr 26. doi:10.1371/journal.pone.0196056
3. Atik A, Stewart T, Zhang J. Alpha-Synuclein as a Biomarker for Parkinson's Disease. *Brain Pathol*. 2016;26(3):410–418. doi:10.1111/bpa.12370
4. Ishii, Ryotaro & Tokuda, Takahiko & Tatebe, Harutsugu & Ohmichi, Takuma & Kasai, Takashi & Nakagawa, Masanori & Mizuno, Toshiki & El-Agnaf, Omar. (2015). Decrease in Plasma Levels of α-Synuclein Is Evident in Patients with Parkinson’s Disease after Elimination of Heterophilic Antibody Interference. PLOS ONE. 10. e0123162. 10.1371/journal.pone.0123162.
5. Stefaniuk CM, Hong H, Harding CV, Maitta RW. (2018) α-Synuclein concentration increases over time in plasma supernatant of single donor platelets. Eur J Haematol. 2018 Jul 28. doi: 10.1111/ejh.13152.
6. Lashuel HA, Overk CR, Oueslati A, Masliah E. The many faces of α-synuclein: from structure and toxicity to therapeutic target. *Nat Rev Neurosci*. 2013;14(1):38–48. doi:10.1038/nrn3406
7. Bougea, A., Stefanis, L., Paraskevas, G.P. et al. Plasma alpha-synuclein levels in patients with Parkinson’s disease: a systematic review and meta-analysis. *Neurol Sci* (2019) 40: 929.
8. Tinsley, R.B., Kotschet, K., Modesto, D., Ng, H., Wang, Y., Nagley, P., Shaw, G., Hormn, M.K. Sensitive and specific detection of α-synuclein in human plasma. *J Neurosci Res* 2010; 88(12) 2693-700
9. Malek N, Swallow D, Grosset KA, Anichtchik O, Spillantini M, Grosset DG. Alpha-synuclein in peripheral tissues and body fluids as a biomarker for Parkinson’s disease-a systematic review. Acta Neurol Scand. 2014;130:59–72. [PubMed]
10. Ishiguro M, Baba H, Maeshima H, Shimano T, Inoue M, Ichikawa T, Yasuda S, Shukuzawa H, Suzuki T, Arai H. Increased Serum Levels of α-Synuclein in Patients With Major Depressive Disorder. The American Journal of Geriatric Psychiatry, 2018, Volume 27, Issue 3, 280 – 286
11. Simonsen A.H, Bahl J.M.C, Danborg P.B, Lindstrom V, Larsen S.O, Grubb A, Heegaard N.H.H, Waldemar G. Pre-analytical factors influencing the stability of cerebrospinal fluid proteins. Journal of Neuroscience Methods, 2013, Volume 215, Issue 2, 234-240

14. Other Information

Please visit our website (www.biosensis.com) for a full range of α-synuclein-related research reagents.

Appendix A: Determining the Number of Microplate Wells Needed for ELISA Experiments

Standard curve, blank and controls:

- Standard (10.0 ng/mL, 5.0 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.625 ng/mL, 0.313 ng/mL, 0.16 ng/mL): 7 wells
- Blank (0 pg/mL): 1 well
- Control vial (either provided in the ELISA kit or the researcher): 1 well

Gives a total of 9 wells used per Standard/Control set.

We recommend that the standard curve, blanks, and controls be run on each separate plate for the most accurate calculations. Note that if two (or more) independent assays are run on one plate, each assay requires its own Standard/Control set. We also recommend that ALL samples (i.e. standards, controls, blanks and test subjects) be performed in duplicate at least.

Thus, for standards, blanks and controls, 9 x 2 = **18 standard wells are required per assay.** This leaves 96 wells – 18 wells = **78 sample wells per plate** for test/patient samples for EACH of our standard 1P ELISA kits run with a standard curve and controls.

1P kit: 78 experimental wells per 96 well ELISA plate experiment
2P kit: 156 experimental wells per 192 well ELISA plate experiment

The 2-Plate Optional Single Control Set:
Some researchers using our 2P kits run a single, duplicate standard curve, blank and control sample set (18 wells) for the entire 2-plate experiment. This allows 78 test/patient samples on the "standard curve plate" (the plate containing the standard curve, controls and blanks) and a full 96 test/patient samples on the "test only plate" for a total available number of 174 test/patients per 2 plate kit. (78 wells +96 wells = 174).

2P kit, single set of controls (in duplicate): 174 experimental wells per 192 well ELISA plate experiment

Example: 60 patient samples, duplicate measurement at one single dilution
How many wells and plates do I need for sixty patients at one dilution with a single sample taken before and after treatment?

1. Calculate the number of test samples: 60 patients, x 2 draws each (e.g. before and after) = 120 stock samples
2. Factor in the number of repeat tests desired, in order to calculate the number of wells required. Biosensis recommends that ALL samples be run in duplicate at least.
3. Multiply the number of stock samples by the number of dilutions per sample. In this example, we will be testing EACH stock sample at a single dilution (i.e. 1:10) in duplicate, so 120 stock samples x 2 wells = 240 microplate wells required for sixty patients. In summary: 60 patients, 4 tests per patient, equals 240 total number of wells required.
4. Decide on the number of standards, blanks and controls run on each plate. We recommend standard, control and blank duplicates for each 96-well plate. Alternatively, one standard, blank and control duplicate set can be run on every 2 plates (2-Plate Optional Single Control Set option discussed above).

Running the Test:
Running the 60 patient experiment with a standard curve, blanks and controls on *each* test plate, in duplicate (our recommended option):

Single Dilution per Test Sample:

(240 wells required) / (78 wells per plate available) = 3.077 plates required, or just over 3 plates, thus researcher will need to order **4 x 1-plate kits or 2 x 2-plate kits** (there will be unused wells) to ensure enough wells for the entire sixty patient sample, tested in duplicate (two draws per patient, 1 dilution, 4 wells per patient, total of 240 wells). The unused 8-well strips can be used for other assays later.

Two Dilutions per Test Sample:

If testing at two dilutions, (e.g. 1:10 & 1:20 per sample) and doing duplicates, then the number of wells/tests is doubled to 480 (e.g. 120 x 2 @ 1:10 = 240, PLUS 120 x 2 @1:20 = 480). Then the number of plates is determined by (480 wells required) / (78 wells per plate available) = 6.15 plates required, or just over 6 plates, thus the researcher will need to order **3 x 2-plate kits and 1 x 1-plate kit** to ensure the minimum number of wells for sixty patient samples tested in duplicate at two dilutions (two draws per patient, 2 dilutions, 8 tests/wells per patient, total of 480 patient tests/wells).

2-Plate Optional Single Control Set Option:

If running 60 patients with a single dilution, and a single duplicate standard curve, blank, and control sample for every two plates, then one has **174 available test wells per 2P kit**.

For the single dilution, sixty patients, 2 draws per patient experiment (240 tests), one would need (240/174) = 1.38 2-plate kits, or **2 x 2-plate kits** would need to be ordered to ensure enough wells for all sixty patient samples.

For the two dilutions per sample, sixty patients, 2 draws per patient experiment (480 tests), one would need (480/174) = 2.76 2-plate kits, or **3 x 2-plate kits** would need to be ordered to ensure enough wells for all sixty patient samples

Appendix B: Tissue Homogenate Preparation Procedure (RIPA Buffer)

The key with any RIPA extraction buffer is to provide enough detergent to solubilize the proteins, but not so much that it will interfere with subsequent uses, particularly native ELISAs where antibody binding is critical. We recommend the following RIPA extraction buffer, which has lower detergent amounts and has been successfully used in brain tissue extracts.

Biosensis In-house RIPA
50 mM Tris-HCL, 150 mM sodium chloride; 2 mM EDTA; 1.0% NP-40 or Triton X-100; 0.5% sodium deoxycholate; pH 7.4 (Note: 0.1% SDS is left out for ELISA samples, but can be added back for western or IP samples, which will be diluted before use). Complete proteinase inhibitor and phosphatase inhibitor cocktails are added to this base buffer in all cases. Tissue samples should be homogenized in ice-cold RIPA buffer, and proper protein preparation procedures including lysates being kept on ice, etc. are required for consistent and best results.

It is recommended that tissue samples should be rapidly excised, weighed, and snap frozen in liquid nitrogen prior to storage at -80°C. We recommended using frozen samples within two weeks of freezing.

RIPA homogenates should be prepared in approximately 10 to 100 volumes of the homogenization buffer to tissue wet weight, but the most appropriate ratio needs to be determined by the user for each tissue. Typical ratios are 10-20 mg wet weight/100 µL of lysate, or 1 g tissue per 10 mL of lysis buffer. The tissue can be homogenized either via sonication or mechanical shearing or both (polytron). The homogenates are centrifuged at ~ 14,000 x g for 30 minutes. The resulting supernatants can be used for ELISAs. Check that sample pH is near 7.4 - 8.0 for best results.

Concentrated stock lysates should be divided into aliquots and frozen at -80°C and thawed and used only once. Lysate stability is fragile and should be used within two weeks for best results.

RIPA Buffer Tissue Lysis and Preparation

1. Sonicate the suspension to homogeneity on ice with a probe sonicator (e.g. Misonix Ultrasonic Liquid Processor). Sonicate in short bursts (5-7 sec) to avoid excessive sample heating
2. Keep homogenates on ice for 30 min, then repeat sonication as well as incubation on ice
3. Centrifuge homogenates for 30 minutes at 10,000 – 20,000 x g and 4°C, then transfer clear supernatants into clean tubes and discard pellets
4. Measure total protein concentration (e.g., BCA or Bradford protein assay)
5. These supernatants may be stored at -80°C and must be centrifuged again for 30 min at 10,000- 20,000 x g and 4°C immediately after thawing and before being used in the ELISA

RIPA Sample Dilution
Prepared, cleared, concentrated, lysates are typically diluted at least 1:4 with Biosensis Sample Diluent A before use in the ELISA. After dilution, check that sample pH is within 7.0 – 8.0 with pH paper for best results.

Note: The final sample dilution will vary depending upon the tissue and exact extraction method. Typically, 1:4 - 1:100 dilution for many, but some tissues can be greater, in order for the assay values to be consistent and fall within the linear range of the assay. Thus, the optimal dilution needs to be determined for each experimental set and laboratory. Spike-recovery experiments are highly recommended.

ELISA results can be reported as ng alpha-synuclein/mg total soluble protein or g wet weight if tissue sample is large enough.

Note: Biosensis recommends evaluating the recovery of alpha-synuclein when preparing tissue extracts. In order to conduct a recovery experiment, known amounts of alpha-synuclein standard are added to an aliquot of the brain tissue homogenates and assayed. Spike-recovery experiments allow comparison of technique and methods. Recoveries of 80-120% of spiked values are acceptable.

Appendix C: Tissue Homogenate Preparation Procedure TBS Buffer

Biosensis In-house Tris-Buffered Saline (TBS)

50 mM Tris-Base and 150 mM sodium chloride pH 7.6. Complete proteinase inhibitor and phosphatase inhibitor cocktails are added to this base buffer in all cases. Tissue samples should be homogenized in ice-cold TBS buffer, and proper protein preparation procedures should be followed, including lysates being kept on ice, etc., to obtain consistent and best results.

It is recommended that tissue samples should be rapidly excised, weighed, and snap frozen in liquid nitrogen prior to storage at -80°C. We recommended using frozen samples within two weeks of freezing.

TBS homogenates should be prepared in approximately 10 to 100 volumes of the homogenization buffer to tissue wet weight, but the most appropriate ratio needs to be determined by the user for each tissue. Typical ratios are 10-20 mg wet weight/100 µL of lysate, or 1 g tissue per 10 mL of lysis buffer. The tissue can be homogenized either via sonication or mechanical shearing or both (polytron). The homogenates are centrifuged at ~ 14,000 x g for 30 minutes. The resulting supernatants can be used for ELISAs. Check that sample pH is near 7.4 - 8.0 for best results.

TBS Buffer Tissue Lysis and Preparation

1. Sonicate the suspension to homogeneity on ice with a probe sonicator (e.g. Misonix Ultrasonic Liquid Processor). Sonicate in short bursts (5-7 sec) to avoid excessive sample heating
2. Keep homogenates on ice for 30 min, then repeat sonication as well as incubation on ice
3. Centrifuge homogenates for 30 minutes at 10,000 – 20,000 x g and 4°C, then transfer clear supernatants into clean tubes and discard pellets
4. Measure total protein concentration (e.g., BCA or Bradford protein assay)
5. These supernatants may be stored at -80°C and must be centrifuged again for 30 min at 10,000 - 20,000 x g and 4°C immediately after thawing and before being used in the ELISA

Concentrated stock lysates should be divided into aliquots and frozen at -80°C and thawed and used only once. Lysate stability is fragile and should be used within two weeks for best results.

TBS Sample Dilution

Prepared, cleared, concentrated, lysates are typically diluted at least 1:4 with Biosensis Sample Diluent A before use in the ELISA. After dilution, check that sample pH is within 7.0 – 8.0 with pH paper for best results.

Note: The final sample dilution will vary depending upon the tissue and exact extraction method. Typically, 1:4 - 1:100 dilution for many, but some tissues can be greater, in order for the assay values to be consistent and fall within the linear range of the assay. Thus, the optimal dilution needs to be determined for each experimental set and laboratory. Spike-recovery experiments are highly recommended.

ELISA results can be reported as ng alpha-synuclein/mg total soluble protein or g wet weight if tissue sample is large enough.

Note: Biosensis recommends evaluating the recovery of alpha-synuclein when preparing tissue extracts. In order to conduct a recovery experiment, known amounts of alpha-synuclein standard are added to an aliquot of the brain tissue homogenates and assayed. Spike-recovery experiments allow comparison of technique and methods. Recoveries of 80-120% of spiked values are acceptable.

Appendix D: Troubleshooting Guide

This α-synuclein ELISA kit has been developed to deliver reproducible results when following the provided assay protocol. Please refer to the troubleshooting guide below when unexpected difficulties are encountered. If you require further assistance, talk to a scientist at Biosensis (biospeak@biosensis.com).

Problem	Cause	Solution
High background (blank OD > 0.30)	Insufficient washing	Make sure to perform 5 washes after each incubation step with sample, detection antibody and streptavidin-HRP conjugate. Follow the recommended manual washing procedure for optimal washing performance
	Excessive concentration of detection antibody and/or HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume.
	Increased incubation time and temperature	Please follow incubation times as stated in the protocol and perform incubations at room temperature
	Contamination	Ensure that all plastic- and glassware used is clean. Do not use the same pipette tip to transfer samples and blank solution into the wells
Low absorbance readings	Concentration of α-synuclein in the sample is lower than the detection limit of this assay	Confirm that the assay protocol was carried out correctly by spiking a known amount of α-synuclein into your sample and/or check that the QC sample value falls within the expected α-synuclein concentration range
	Insufficient antibody or insufficient HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume
	Reagents expired	Ensure correct storage of the kit and do not use the kit beyond its expiry date

Problem	Cause	Solution
Low absorbance readings	Decreased incubation times and temperature	Please follow incubation times as stated in the protocol and warm reagents to room temperature before use
	Microplate wells dried out	Do not leave microplate wells without solution for an extended time
	Wash buffer not diluted	Ensure that the wash buffer is diluted with ultrapure water to a 1x working solution
	Stop solution not added	Add the stop solution to obtain a yellow reaction product that can be measured at 450 nm
Standard OD values above plate reader limit	Excessive incubation with TMB substrate solution	Reduce incubation time by stopping the reaction at an earlier time-point
Sample OD values above standard curve range	α-synuclein concentration in sample is too high	Perform a serial dilution of your sample to obtain a reading that falls within the standard curve
High coefficient of variations (CV)	TMB and stopping solution not added consistently	Add TMB and stopping solution to the wells in the same order and speed
	Inconsistent pipetting	Ensure pipettes are working correctly and are calibrated; review your pipetting technique; add standards and samples to the wells using a single-channel rather than a multi-channel pipette
	Insufficient mixing of reagents	Briefly vortex to mix solutions before pipetting into the wells
	Bottom of the plate is dirty	Clean the bottom of the plate before reading the plate