



LEGEND MAX™ Human α -Synuclein ELISA Kit Protocol

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Sam Li¹

¹BioLegend

BioLegend

Tech. support email: tech@biolegend.com



Sam Li

BioLegend

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Guidelines

Notes: Allow kit components to be brought to room temperature before use.

FAQ:

- **The protocol says to shake the plate overnight at 2-8°C. What if my lab doesn't have this capability?**

The assay is optimized for the overnight incubation step to shake at 2-8°C . If shaking is not available, results should be acceptable however overall signal may be lower which could impact signal to noise ratio. Please keep this in mind when interpreting data.
- **What wavelength do I use to read the plate?**

Since this is a chemiluminescent assay, there is no wavelength to read the plates. The plate reader should have a hole setting in the cartridge where the light emitted by the substrate can pass through and be detected. Wavelength settings are for colorimetric assays only.
- **I am getting very high background and my standard curve is not linear at the low end. What is causing this?**

This is typically a washing issue. Inadequate washing will lead to high background and poor sensitivity at the low end of the curve. To fix the issue, either use a plate washer or squirt bottle for all washing steps. Pipette washing will not be vigorous enough. This should help reduce the background and in turn give better sensitivity at the low end.
- **I have a plateau at the top end of my standard curve, what could be causing this?**

Typically this is caused by not preparing your standard curve from intermediate #2 and using the reconstituted standard instead. The concentration is too high to be detected by the reader and will cause a plateau of the first few points on the standard curve.
- **My samples are undetectable in the assay, what is causing this?**

The dilution factor you chose might have been too high. Try running your samples more concentrated to see if they are detectable. If you run them undiluted and they are still undetectable, the level of alpha-Synuclein in your samples is too low to be detected by this kit and you may need to concentrate them prior to testing them.
- **What are the recommended dilutions for my samples?**

This is going to vary based on your specific samples. We recommend running 2-3 different dilutions for each sample, starting with a minimum of 1:10 dilution. This should help ensure one of the dilutions will fall within the range of the standard curve. If you are testing a large number of samples, consider doing a trial run with a subset of samples to optimize the dilution factors and determine if any matrix effects exist. This is a critical step when running a large number of samples and failure to take the necessary optimization steps can result in inconclusive data.
- **What types of samples will work with this assay?**

Currently, this assay has been tested with CSF, plasma, saliva, whole blood and cell lysates.
- **Does this kit detect gamma or beta synuclein and is it cross reactive with other species?**

This kit is specific for human alpha synuclein. It does not detect beta or gamma synuclein and does not cross



react with rodent.

▪ **What if I have further questions?**

Please call our technical support at 1.877.273.3103 or send e-mail to tech@biolegend.com. We will be more than happy to answer any questions you may have.

Materials

Kit Components:

- Capture Antibody Coated Plate* (1 plate)
- α -Synuclein Standard (1-25 μ g vial)
- 5X Wash Buffer (250mL)
- 2X Reagent Diluent (32mL)
- Biotinylated Primary Antibody (25 μ L)
- Streptavidin-HRP (25 μ L)
- Chemiluminescent Substrate A (6mL)
- Chemiluminescent Substrate B (6mL)
- Plate Sealer (3)

* The strips in this plate are not readily removable; it is intended to be used as a whole plate. Attempting to remove the strips will result in damage for which BioLegend is not responsible.

Storage: Store kit at 2-8° C. We suggest storing the α -Synuclein Standard component only at $\leq$ -70° C if you do not intend to use the kit within 2 weeks of receipt.



I. Preparation of 1X Wash Buffer

- 1 Label a 1L bottle as "1X Wash Buffer".
- 2 Dilute 5X Wash Buffer 1:5 using lab grade water* and mix well.*Note: Lab grade filtered water such as injection grade, cell culture grade, Reverse Osmosis De-Ionization (RODI).

II. Preparation of 1X Reagent Diluent

- 3 Label an appropriate sized bottle as "1X Reagent Diluent".
- 4 Dilute 2X Reagent Diluent to 1X by adding 30mL of 2X Reagent Diluent to 30mL of lab grade water in the bottle labeled "1X Reagent Diluent".
- 5 Mix well by vortex.

III. Preparation of Standard Intermediates (Refer to Table 1)

- 6 Reconstitute lyophilized standard with 50 μ L of lab grade water and mix well by vortex. The concentration after reconstitution will be 500 μ g/mL.
- 7 Label (2) 1.5mL microcentrifuge tubes as intermediate #1 & intermediate #2.
- 8 Aliquot 990 μ L of 1X Reagent Diluent into intermediate #1 & intermediate #2 tubes.
- 9 Remove 10 μ L from the vial of reconstituted standard and add to 990 μ L of 1X Reagent Diluent in intermediate tube #1. Mix well by vortex.
- 10 Remove 10 μ L from intermediate #1 and add to 990 μ L of 1X Reagent Diluent in intermediate #2 tube. Mix well by vortex.
- 11 The final concentration of intermediate tube #2 will be 50ng/mL.



TABLE 1. Preparation of Standard Intermediates

Tube Number	Volume of Standard	Volume of 1X Reagent Diluent (μL)	Final Concentration (ng/mL)
Intermediate #1	10 μL of reconstituted standard	990	5,000
Intermediate #2	10 μL of intermediate #1	990	50

IV. Preparation of Standard Curve (Refer to Table 2)

- 12 Label (8) 1.5mL microcentrifuge tubes as #1-8.
- 13 Add 1280 μL of 1X Reagent Diluent to tube #1 and 825 μL of 1X Reagent Diluent to tubes #2-8.
- 14 Remove 40 μL from intermediate #2 and add to 1280 μL of 1X Reagent Diluent in tube #1.
- 15 Mix well by vortex (this will be the top point of the standard curve, 1500pg/mL).
- 16 Remove 550 μL from tube #1 and add to 825 μL of 1X Reagent Diluent in tube #2.
- 17 Mix well by vortex.
- 18 Continue making 2.5 fold serial dilutions by adding 550 μL of the previous dilution to 825 μL of 1X Reagent Diluent in tubes #3-7. Mix well by vortex between each dilution. Note: Tube #8 will be the zero or blank sample and should only contain 1X Reagent Diluent.



TABLE 2. Preparation of Standard Curve

Tube Number	Volume of Standard (μL)	Volume of 1X Reagent Diluent (μL)	Final Concentration (pg/mL)
1	40 μL intermediate #2	1280	1500
2	550 μL of #1	825	600
3	550 μL of #2	825	240
4	550 μL of #3	825	96.0
5	550 μL of #4	825	38.4
6	550 μL of #5	825	15.4
7	550 μL of #6	825	6.1
8	0	825	0

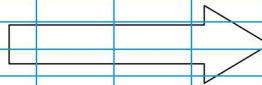
VI. Running the Assay - Day 1

- 19 Remove the plate from the foil pouch.
- 20 Add 300 μL/well of 1X Wash Buffer.
- 21 Dump out wash buffer and pat dry.
- 22 Repeat 3 more times for a total of 4 washes.
- 23 Add 200 μL of each standard to the plate in duplicate or triplicate. Follow the plate layout below. Note: The volumes in Table 2 are sufficient for running the standard curve in triplicate.
- 24 Add 200 μL of each sample dilution to the plate.
- 25 Cover the plate with the plate sealer provided.
- 26 Incubate overnight at 2–8° C, while shaking.



TABLE 3.

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



VII. Running the Assay - Day 2

27 Notes: Allow kit components to be brought to room temperature before use.

1. Preparation of Biotinylated Primary Antibody.

27.1 Label a 15mL centrifuge tube as "Biotinylated Primary Antibody".

27.2 Dilute the biotinylated primary antibody by adding 6 μ L* of the Biotinylated Primary antibody stock to 6mL of 1X Reagent Diluent in the 15mL tube labeled "Biotinylated Primary Antibody"*Note: A quick spin in a centrifuge is suggested prior to pipetting to ensure liquid is at the bottom of the vessel.

27.3 Mix well by vortex.

28 Remove plate from refrigerator and dump contents.

29 Add 300 μ L/well of 1X Wash Buffer.

30 Dump out wash buffer and pat dry.

31 Repeat 3 more times for a total of 4 washes.

32 Add 50 μ L/well of the Biotinylated Primary Antibody to the plate (prepared above).

33 Cover and incubate for 2 hours at room temperature.



34 Preparation of Streptavidin HRP (Refer to Table 4)

34.1 Label a 1.5mL microcentrifuge tube as "HRP Intermediate".

34.2 Label a 50mL tube as "Diluted Streptavidin HRP".

34.3 Make the "HRP Intermediate" by adding 10 μ L* of Streptavidin-HRP stock to 990 μ L of 1X Reagent Diluent in the 1.5mL microcentrifuge tube labeled "HRP Intermediate".*Note: A quick spin in a centrifuge is suggested prior to pipetting to ensure liquid is at the bottom of the vessel.

34.4 Mix well by vortex.

34.5 Remove 150 μ L from the HRP Intermediate tube and add to 22.35mL of 1X Reagent Diluent in the 50mL tube labeled "Diluted Streptavidin HRP".

34.6 Mix well by vortex.

TABLE 4.

TubeName	Volume Streptavidin-HRP stock (mL)	Volume of 1X Reagent Diluent (mL)	Dilution Factor
HRP Intermediate	0.01	0.990	100
DilutedStreptavidin HRP	0.150 (intermediate)	22.35	150

35 Remove plate from incubation and dump contents.

36 Add 300 μ L/well of 1X Wash Buffer.

37 Dump out wash buffer and pat dry

38 Repeat 3 more times for a total of 4 washes.



- 39 Add 200 μ L/well of Diluted Streptavidin HRP to the plate (prepared above).
- 40 Cover and incubate for 1 hour at room temperature.
- 41 Remove plate from incubation and dump contents.
- 42 Add 300 μ L/well of 1X Wash Buffer.
- 43 Dump out wash buffer and pat dry.
- 44 Repeat 3 more times for a total of 4 washes.
- 45 Mix chemiluminescent substrates for use:
 - 45.1 Add 5.5mL of substrate A to a 15mL centrifuge tube.
 - 45.2 Add 5.5mL of substrate B to the same 15mL centrifuge tube.
 - 45.3 Mix well by vortex.
- 46 Add 100 μ L of mixed substrate per well.

Note: If reading multiple plates add substrate one plate at a time; do not add substrate to all plates at the same time. Add substrate to each plate immediately before reading.
- 47 Shake plate on either a plate shaker or using the shaking mechanism within the plate reader for 10-15 seconds.
- 48 Read plate immediately.

Note: The recommended luminometer settings are to read at a mid-range sensitivity level for 1 second per well. These settings will vary between plate reader manufacturers, please consult your owner's manual prior to performing this assay.

Suggested Settings for Biotek Synergy2 Plate Reader*

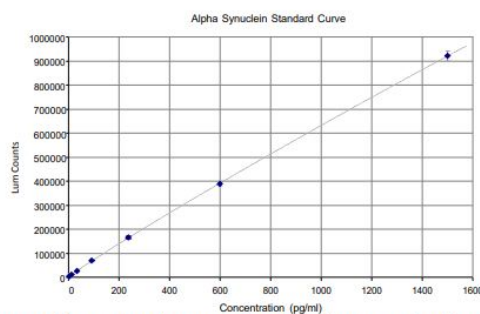
Function	Setting
Detection Method	Luminescence
Read Type	Endpoint
Integration Time	1 second
Emission	Hole
Optics Position	Top
Sensitivity	110

*Settings for other plate readers unknown.

Consult your owners manual prior to starting the assay to determine the optimal settings for your instrument

Data:
Standard Curve

Standard Concentration	Average Lum Counts N=3	Std Dev	% CV
1500.0	923,788	42,521	5%
600.0	380,374	3,051	1%
240.0	152,353	2,818	2%
96.0	62,177	3,875	6%
38.4	25,841	1,123	4%
15.4	11,720	531	5%
6.1	6,046	281	5%
0.0	3,011	119	4%

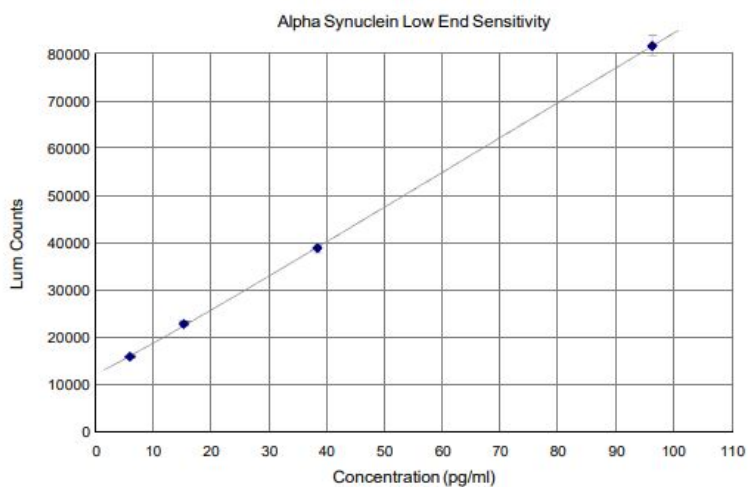


Note: Raw luminescent counts will vary between plate readers, however, the dynamic range should not be affected by these differences.



4-Parameter Curve Fit- recommended however linear regression could also be used

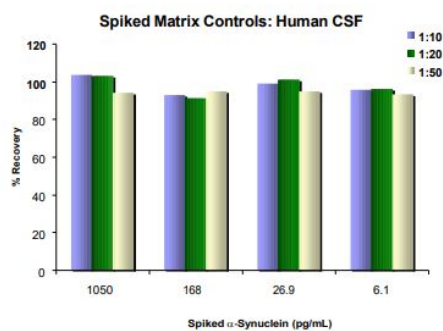
CurveFormula	A	B	C	D	R2
$Y = (A-D)/(1+(X/C)^B) + D$	3.08E+03	1.06	2.02E+03	1.82E+06	1



Data (continued):

Cross Reactivity Curve

Standard Concentration	Avg Lum Counts α -Synuclein	Avg Lum Counts β -Synuclein	Avg Lum Counts γ -Synuclein
1500.0	1,199,195	5,972	3,669
600.0	506,624	4,519	3,486
240.0	213,564	3,872	3,513
96.0	89,262	4,052	3,812
38.4	39,044	3,841	3,780
15.4	16,599	3,972	3,971
6.1	8,670	4,008	3,969
0.0	4,024	3,852	4,197



Minimum Required Dilution (MRD) and Dilutional Linearity

Matrix	Dilution	Calculated Conc. Adjusted for Dilution	Std Dev	%CV	% Recovery to MRD
CSF pg/mL	1:10	1826.5	24.2	1.3%	MRD
	1:20	1794.3	26.1	1.5%	98.2%
	1:50	1707.2	203.8	11.9%	93.5%
Saliva ng/mL	1:25	28.0	0.76	0.3%	MRD
	1:50	27.5	0.580	2.1%	98.1%
	1:100	28.6	0.59	0.2%	101.9%
Whole Blood ug/mL	1:200,000	132.0	12.4	9.4%	MRD
	1:400,000	134.7	9.5	7.0%	102.0%
	1:800,000	140.2	2.2	1.5%	106.2%