ADNI Alzheimer's Disease Neuroimaging Initiative

Human PS129 Luminex Assay in Cerebrospinal Fluid

Zhang Lab, University of Washington; expanded from Wang et al, Sci Transl Med 4, 121ra120, 2012[1]

Summary (or Abstract)

This bead-based immunoassay, built upon the Luminex platform is designed to accurately and reproducibly determine the concentration of alpha-synuclein species post-translationally modified by phosphorylation at serine 129 (pS129) in the biofluid cerebrospinal fluid (CSF).

Materials and Method

Materials

1. Luminex MicroPlex Microspheres, 1 mL (Luminex, e.g., catalog # LC10061-01 for Region 61). Beads are activated before coating by using Sulfo-NHS and EDC according to Luminex's protocol.
2. Capture antibody beads (10×): Coat 20 µg high affinity rabbit anti-alpha-synuclein antibody ASY-1 (gift from Dr. Poul H. Jensen, University of Aarhus, Denmark) to 2.5×10^5 activated beads (4°C ~20h, then R.T. 2-3h) following Luminex's instructions. Store as 10× stock in 200 µl of 1% BSA/PBS at 4°C.
3. Biotinylated detection antibody (100×): mouse anti-human pS129, clone P-syn/81A, developed by our collaborators at the University of Pennsylvania, USA[1]; now available from BioLegend. Antibodies are biotinylated using Pierce EZ-Link™ Sulfo-NHS-LC-Biotin, and then stored at –80°C in aliquots (100 ng/µL).
4. PS129 Standard (1µg/µL): generated by phosphorylation of human recombinant α-synuclein with casein kinase 2 and purification with HPLC[1]; stored at –80°C in one-time use aliquots.
5. Streptavidin-R-Phycoerythrin (RPE) (1000×): Prozyme, catalog # PJ31S, 1 mg/mL; stored at 4°C.
6. Assay Diluent: 0.1% BSA (Sigma, catalog # A-9647) in PBS (pH7.4), stored at 4°C. Can be diluted from a 1% BSA/PBS stock solution before use.
7. Wash Solution Concentrate (20x), 1 L:

   Sodium phosphate dibasic Na_2HPO_4 (FW142) 23.5 g

Sodium phosphate monobasic NaH_2PO_4 (FW120)	4.56 g
Sodium chloride NaCl (FW 58.5)	175.4 g
Sodium azide NaN_3	1 g
Tween-20	20 mL
Triton X-100	20 mL

Adjust pH to 7.4, add water to 1 L and stir for at least 30 minutes at RT. Store at R.T. or aliquot into 25 ml/tube and keep at 4°C.

8. 96 well Filter Plate (Millipore, catalog # A-9647 MSBVN-1250)

Sample preparation

1. Thaw samples [CSF containing 10% protease inhibitor cocktail (Sigma cat# P2714; prepared in 10 mL of H_2O)] on wet ice.
2. Centrifuge samples at 15000 x g for 10 min at 4˚C.
3. Add 25 µL 0.1% BSA in PBS to wells first, then add 75 µL CSF. The final volume of the well will be 100 µL. The dilution factor for the CSF sample will be 1.47 (1.1*1.33).

Preparation of standards

Thaw the pS129 protein standard stock on ice within one hour of performing the assay.

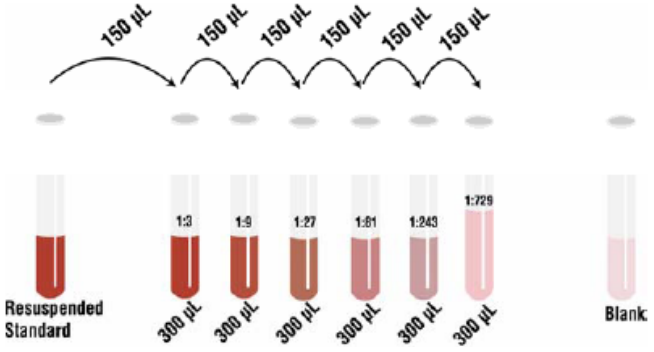
Prepare the standard curves

The standard curve is made by serially diluting the thawed pS129 protein standard stock in Assay Diluent (0.1% BSA in PBS). See diagram below. Discard all remaining reconstituted and diluted protein standards after completing the assay.

Concentrations of pS129 protein standard (ng/mL):

STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9
20	6.667	2.222	0.741	0.247	0.082	0.027	0.009	0.003

PS129 STD stock is 1 µg/µL. Take 1 µL of original STD, and add 999 µL Assay Diluent to make a 1 µg/mL solution (STD0). Take out 20 µL of STD0, and add 980 µL Assay Diluent to make a 20 ng/mL solution - this is STD1.



Preparation of antibody conjugated beads

Determine the number of wells required for the assay. Standard curves and samples may be run singly or in replicates, as desired. The beads are provided as a 10x concentrate. Immediately before dispensing, vortex the 10x bead concentrate for 1 minute, then sonicate for 1 minute. To make a 1x capture bead stock, mix 1 part 10x bead concentrate with 9 parts 1x Working Wash Solution. Each well requires **25 µL** of the diluted beads. Diluted beads should be used immediately, but may be stored overnight at 2 to 8°C when necessary.

Assay Procedure

1. Pre-wet the wells designated for the assay. Pipette 0.2 mL of 1x Working Wash Solution into designated wells. Wait 15 to 30 seconds then aspirate the Working Wash Solution from the wells using the vacuum manifold.
2. Vortex the diluted bead solution for **1 minute**, then sonicate for **1 minute** and vortex the diluted bead solution for **1 minute** immediately prior to use in the assay.
3. Pipette 25 µL of the diluted bead solution into each well. Once the beads are added to the plate, keep the plate shielded from light.
4. Add 0.2 mL Working Wash Solution to the wells. Allow the beads to soak for 10 seconds, then remove the Working Wash Solution from the wells by aspiration with the vacuum manifold. Repeat this washing step. Blot residual liquid from the bottom of the plate on clean paper towels.
5. Pipette 100 µL standards into designated wells; use 100 µL of Assay Diluent as blank/background.
6. For sample wells, add 25 µL 0.1% BSA in PBS to wells first, then add 75 µL CSF.
7. Incubate the plate for **18 hours** at 4˚C on an orbital shaker set to 600 rpm to maintain bead suspension during incubation.
8. At the end of the incubation, remove the liquid from the wells by aspiration with a vacuum manifold (DO NOT EXCEED 5 inches Hg). Add 0.2 mL Working Wash Solution to the wells. Allow the beads to soak for 10 seconds, then aspirate with the vacuum manifold. Repeat this washing step two more times for a total of three washes**.**
9. Ten to fifteen minutes before the end of the incubation, prepare the Detector Antibody 1x working solution. The Detector Antibody is stocked as a 100x concentrate (100 ng/µL). To make a 1x Detector Antibody stock for a single analyte assay, mix 1 part 100x Detector Antibody Concentrate with 99 parts Assay Diluent (0.1% BSA in PBS). Each well requires 100 µL of the diluted Detector Antibody. Into all wells, pipette 100 µL of the 1x Biotinylated Detector Antibody.
10. Incubate the plate for **3 hours** at room temperature on an orbital shaker. Shaking should be sufficient to keep the beads suspended during the incubation (600 rpm).

11. At the end of the incubation, remove the liquid from the wells by aspiration with a vacuum manifold (DO NOT EXCEED 5 inches Hg). Add 0.2 mL Working Wash Solution to the wells. Allow the beads to soak for 10 seconds, then aspirate with the vacuum manifold. Repeat this washing step two more times for a total of three washes**.**
12. Ten to fifteen minutes before the end of the incubation, prepare the Streptavidin-RPE 1x working solution. The conjugate is provided as a 1000x concentrate (1 mg/ml). Dilute 1 part Streptavidin-RPE 1000x Concentrate with 999 parts Assay Diluent (0.1% BSA in PBS). Each well requires 100 µL of prepared 1x Streptavidin-RPE. The Streptavidin-RPE 1x Working Solution Conjugate must be prepared fresh for each assay and be protected from light.
13. Add 100 µL of prepared Streptavidin-RPE 1x Working Solution to each well.
14. Incubate the plate for **30 minutes** at room temperature on an orbital shaker. Shaking should be sufficient to keep beads suspended during the incubation (600 rpm).
15. Prepare the Luminex 200™ instrument during this incubation step.
16. After the 30 minute incubation, remove the liquid from the wells by aspiration with the vacuum manifold (DO NOT EXCEED 5 inches Hg). Add 0.2 mL Working Wash Solution to the wells. Allow the beads to soak for 10 seconds, then aspirate with the vacuum manifold. Repeat this washing step three more times for a total of **four washes**. Blot residual liquid from the bottom of the plate on clean paper towels.
17. Add 100 µL of Working Wash Solution to each well. Shake the plate on an orbital shaker (600 rpm) for 3 to 5 minutes to resuspend the beads. The assay should be read on the same day to prevent signal degradation.
18. Uncover the plate; insert the plate into the XY platform of the Luminex 200™ instrument, and analyze the samples.

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This document was derived from our published homebrew assay. [1] It was updated on 5/28/2015 by Hua Wang for implementation of this study. The addition of a protein standard at 0.003 ng/ml was the only substantial modification of the original protocol.

Dataset Information

This methods document applies to the following dataset(s) available from the ADNI repository:

Dataset Name	Date Submitted
160914 UW – Zhang Lab, tot asyn, ps129, hgb	14 September 2016

References

1 *Wang, Y. et al. Phosphorylated alpha-synuclein in Parkinson's disease. Science translational medicine **4**, 121ra120, doi:10.1126/scitranslmed.3002566 (2012).*

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