ADNI Alzheimer's Disease Neuroimaging Initiative

Human CSF C3 FH PROTOCOL

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Summary

CSF C3 and FH levels were measured using a multiplex human neurodegenerative kit (HNDG1-36K; Millipore, Billercia, MA) according to the manufacturer's over-night protocol with minor modifications. Briefly, CSF samples were thawed on ice, treated 1:1 with 2× RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 1 mM PMSF (Sigma-Aldrich, St. Louis, MO), incubated on ice for 1 hr, then centrifuged at 15 000 g for 10 minutes at 4 °C. Samples were further diluted 1:800 with 0.1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) pH7.4 and 50 ul of diluted sample was used per well. Plates were analyzed using a LiquiChip Luminex 200TM Workstation (Qiagen, Valencia, CA) [1].

Materials & Instruments

1. Milliplex^{TM} MAP Human neurodegenerative kit (C3 FH multiplex; HNDG1-36K; Millipore, Billercia, MA) containing:

Reagents	
10x Wash Buffer (contains 0.05% Proclin)	dilute with Milli-Q water (dilute only volume needed for assay)
Assay Buffer	ready to use
Quality Control 1	reconstitute to stocked solution with 250ul Milli-Q water aliquots stored at -80 ^{O}C
Quality Control 2	reconstitute to stocked solution with 250ul Milli-Q water aliquots stored at -80 ^{O}C
Anti-Human Complement C3 Bead (20×) Anti-Human Complement Factor H Bead (20x)	dilute to 1x with bead diluent (dilute only volume needed for assay)
Human Neurodegenerative Disease Panel 1 Standard (stock: C3 200ng/ml, FH	reconstitute to stocked solution with 250ul Milli-Q water

1000ng/ml)	aliquots stored at -80 °C
Bead Diluent	ready to use
Detection Antibody	ready to use
Streptavidin-PE	ready to use
Luminex filter plate	ready to use

2. 0.1% BSA (Sigma-Aldrich, St. Louis, MO; A-9647) in PBS pH7.4
3. 2× RIPA-PIC-PMSF (100 ml)

Stock	amount	final concentration
1M NaCl	30 mL	300 mM
NP-40	2 mL	2%
Sodium deoxycholate	1 g	1%
10% SDS	2 mL	0.2%
400 mM Tris (pH8.0)	25 mL	100 mM

   Add water to 100 mL. Store at 4°C or -20°C. Immediately before use, add Protease Inhibitor Cocktail (PIC; Sigma-Aldrich, St. Louis, MO; P2714) and PMSF (Sigma-Aldrich, St. Louis, MO; P7626) so that final PIC is 1× and PMSF is 1 mM (for example, 9 mL 2X RIPA + 1 mL 10X PIC + 40µl 250 mM PMSF)

4. LiquiChip System Fluid 10X (Qiagen, Valencia, CA; 922902)
5. Plate shaker
6. Sonicator
7. Vacuum filtration unit
8. LiquiChip Luminex 200TM Workstation (Qiagen, Valencia, CA)

Sample Preparation

1) Thaw CSF samples on ice.

2) Add 20ul of 2×RIPA-PMSF to 20ul of CSF, vortex to mix; then incubate 1 hour on ice.

3) Centrifuge 15000 × g for 10 min at 4°C.

4) Transfer 8ul of CSF+RIPA supernatant to 1592ul of 0.1%BSA/PBS (1:400 dilution) in a new 2-ml tube on ice. Vortex to mix.

5) Add 100ul of 1:400 diluted CSF solution to 100ul of 0.1% BSA/PBS (1:1 dilution). Vortex to mix, then use 50ul of diluted CSF per well. Alternatively, add 25ul of 1:400 diluted CSF solution

and then 25ul of 0.1% BSA/PBS per well directly. (Note: The actual final dilution is 1:800. But since 25ul of standards will be used, the dilution factor for calculation should be 400*1.1.)

Preparation of Standard and Quality Controls

Reconstitute standard with 250ul Milli-Q water; mix by inverting the vial several times and/or gentle vortexting. Allow the vial to sit on ice for 10 minutes before use. The stock solution can be aliquoted and stored at -80 ^{O}C. The quality controls should be prepared the same way.

Preparation of working standards: The standard curve is made by serially diluting (1:4) the reconstituted standard in **assay buffer** (e.g., transfer 50µL of STD1 to 150ul of assay buffer to make STD2, and so on). Standard dilutions may be performed in glass or plastic tubes. The diluted standards should be used within 1 hour.

Discard all remaining diluted standards after completing the assay.

Concentrations of standard (ng/mL) for C3 and FH:

	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
C3	200	50	12.5	3.125	0.781	0.195	0.049	0.0122
FH	1000	250	62.5	15.625	3.906	0.977	0.244	0.061

Bead Preparation

Dilute 20x beads to 1x with bead diluent

Vortex 20x beads for 60s, sonicate for 30s, vortex for 60s

Take for example 30ul C3 beads + 30ul FH beads + 540ul bead diluent (dilute only volume of beads needed on day of assay, store remaining 20x beads at 4 ^{O}C).

Protect from light.

Assay Procedure

Day 1

1. Allow reagents to warm to room temperature.
2. Pre-wet the wells designated for the assay. Pipette 0.2 mL of assay buffer into designated wells. (Optional: incubate the plate at R.T. on an orbital shaker (300-600 rpm) for 10 minutes.) Wait 15 to 30 seconds and then aspirate the assay buffer from the wells using the vacuum manifold (DO NOT EXCEED 5 inches Hg). Wells not used during the assay should be kept dry for future use. Do NOT to use an adhesive plate cover to seal the unused wells. Blot residual liquid from the bottom of the plate on clean paper towels and wipe with Kimwipes.
3. For standards and QC samples, pipette 25 µL plus 25ul of assay buffer into the appropriate wells (add 50ul of assay buffer to the background well(s)).
4. For CSF samples, pipette 50µL of diluted (in 0.1%BSA/PBS) CSF into the appropriate wells
5. Vortex the diluted bead solution for 60 seconds, then sonicate for 30 seconds, vortex 60 seconds immediately prior to use in the assay.
6. Add 25ul 1× mixed beads (vortex after every 8 wells) into each well. Total volume in all wells: 75ul.
7. Incubate the plate overnight (~18 hours) in cold room on an orbital shaker. Shaking should be sufficient to keep beads suspended during the incubation (600 rpm).

Day 2

8. Allow reagents to warm to room temperature.
9. 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 each well. 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. Blot residual liquid from the bottom of the plate on clean paper towels and wipe with Kimwipes.
10. Into all wells, pipette 25 µL of the 1× Detection Antibody.
11. 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). DO NOT ASPIRATE.

12. Add 25 µL Streptavidin-PE to each well.

15. Incubate the plate for 30 minutes at room temperature on an orbital shaker. Shaking should be sufficient to keep beads suspended during the incubation (600rpm).

16. Prepare the Luminex 200™ instrument during this incubation step.

17. After the 30 minutes 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 two more times for a total of three washes. Blot residual liquid from the bottom of the plate on clean paper towels and wipe with Kimwipes.

18. Add 100 µL of Working Wash Solution to each well. Shake the plate on an orbital shaker (600 rpm for 5 minutes) to resuspend the beads. The assay should be read on the same day to prevent loss of beads and fluorescent intensity.

19. Uncover the plate; insert the plate into the XY platform of the Luminex 200™ instrument and analyze the samples.

Dataset Information

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

Dataset Name	**Date Submitted**
Zhang Lab – CSFC3FH	09 July 2013

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References

1. Wang Y, Hancock AM, Bradner J, Chung KA, Quinn JF, Peskind ER, Galasko D, Jankovic J, Zabetian CP, Kim HM, Leverenz JB, Montine TJ, Ginghina C, Edwards KL, Snapinn KW, Goldstein DS, Shi M, Zhang J (2011) Complement 3 and factor h in human cerebrospinal fluid in Parkinson's disease, Alzheimer's disease, and multiple-system atrophy. The American journal of pathology 178 (4):1509-1516. doi:10.1016/j.ajpath.2011.01.006