6-7-2023

Standardized immunoprecipitation protocol for efficient isolation of native apolipoprotein E particles utilizing HJ15.4 monoclonal antibody

Justin O'Leary
Mayo Clinic

Ana-Caroline Raulin
Mayo Clinic

Zonghua Li
Mayo Clinic

Yuka Martens
Mayo Clinic

Yasuteru Inoue
Mayo Clinic

See next page for additional authors

Recommended Citation

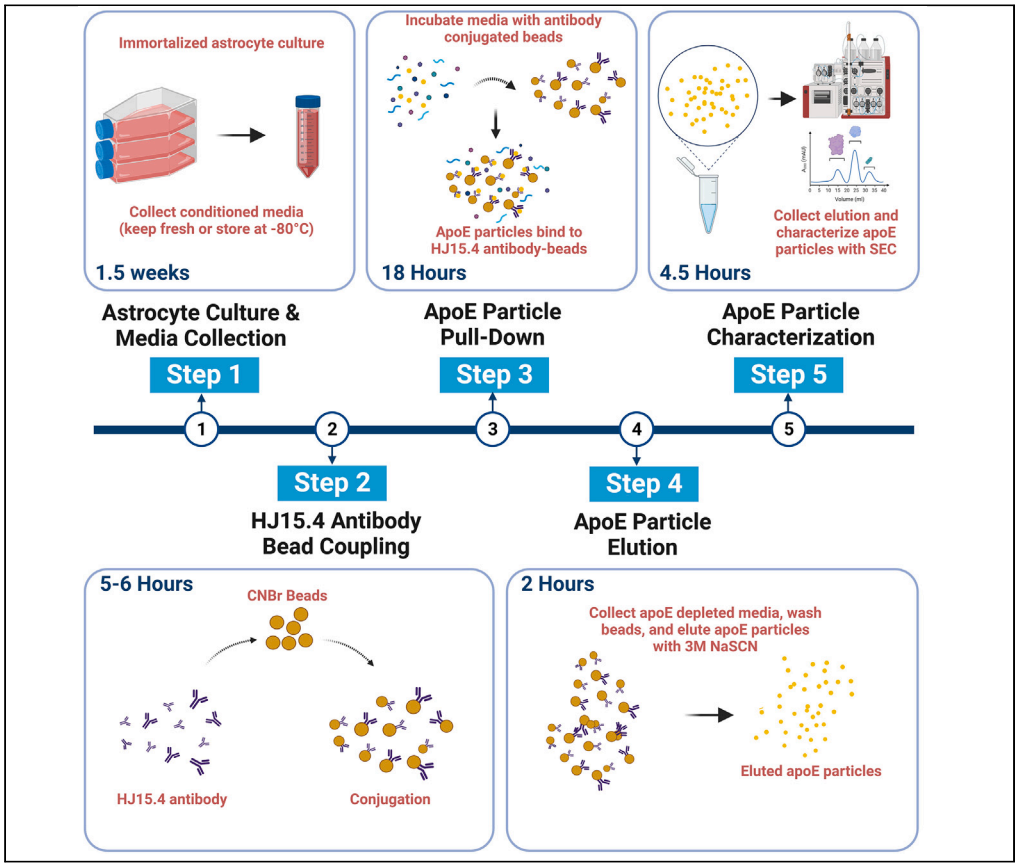
O'Leary, Justin; Raulin, Ana-Caroline; Li, Zonghua; Martens, Yuka; Inoue, Yasuteru; Strickland, Michael R; Han, Xianlin; Holtzman, David M; Bu, Guojun; and Zhao, Na, "Standardized immunoprecipitation protocol for efficient isolation of native apolipoprotein E particles utilizing HJ15.4 monoclonal antibody." STAR Protocols. 4, 2. 102271 (2023).
https://digitalcommons.wustl.edu/oa_4/2789

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Standardized immunoprecipitation protocol for efficient isolation of native apolipoprotein E particles utilizing HJ15.4 monoclonal antibody



The apolipoprotein E protein (apoE) confers differential risk for Alzheimer's disease depending on which isoforms are expressed. Here, we present a 2-day immunoprecipitation protocol using the HJ15.4 monoclonal apoE antibody for the pull-down of native apoE particles. We describe major steps for apoE production via immortalized astrocyte culture and HJ15.4 antibody bead coupling for apoE particle pull-down, elution, and characterization. This protocol could be used to isolate native apoE particles from multiple model systems or human biospecimens.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Justin O'Leary, Ana-Caroline Raulin, Zonghua Li, ..., David M. Holtzman, Guojun Bu, Na Zhao

oleary.justin@mayo.edu (J.O.)
zhao.na@mayo.edu (N.Z.)

Highlights

APOE3/3 immortalized astrocyte culture conditions and conditioned media collection

Detailed two-day immunoprecipitation protocol isolating native apoE lipoprotein particles

Characterization of native apoE lipoprotein particle size distribution and lipid profile

Protocol applicable to multiple apoE lipoprotein particle sources and downstream assays

O'Leary et al., STAR Protocols 4, 102271
June 16, 2023
https://doi.org/10.1016/j.xpro.2023.102271

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Standardized immunoprecipitation protocol for efficient isolation of native apolipoprotein E particles utilizing HJ15.4 monoclonal antibody

Justin O'Leary,[1,5,*] Ana-Caroline Raulin,[1,5] Zonghua Li,[1] Yuka Martens,[1] Yasuteru Inoue,[1] Michael R. Strickland,[2] Xianlin Han,[3,4] David M. Holtzman,[2] Guojun Bu,[1] and Na Zhao[1,6,*]

[1]Department of Neuroscience, Mayo Clinic, Jacksonville, FL 32224, USA

[2]Department of Neurology, Hope Center for Neurological Disorders, Knight Alzheimer's Disease Research Center, Washington University in St. Louis, St. Louis, MO, 63110, USA

[3]Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA

[4]Barshop Institute for Longevity and Aging Studies, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA

[5]Technical contact

[6]Lead contact

*Correspondence: oleary.justin@mayo.edu (J.O.), zhao.na@mayo.edu (N.Z.)
https://doi.org/10.1016/j.xpro.2023.102271

SUMMARY

The apolipoprotein E protein (apoE) confers differential risk for Alzheimer's disease depending on which isoforms are expressed. Here, we present a 2-day immunoprecipitation protocol using the HJ15.4 monoclonal apoE antibody for the pull-down of native apoE particles. We describe major steps for apoE production via immortalized astrocyte culture and HJ15.4 antibody bead coupling for apoE particle pull-down, elution, and characterization. This protocol could be used to isolate native apoE particles from multiple model systems or human biospecimens.

BEFORE YOU BEGIN

Protocol overview

The three alleles of the apolipoprotein E gene (*APOE*), ε2, ε3, and ε4, confer a differential risk profile for the development of Alzheimer's disease (AD) with ε4 significantly increasing risk and ε2 having a protective effect.[1,2] These alleles encode the three apoE isoforms, apoE2, apoE3, and apoE4, which are the primary mediators of lipid transport between cells in the central nervous system.[3] It has been hypothesized that the biochemical and biophysical properties of these apoE lipoprotein particles from different isoforms lead to differential effects on cellular lipid homeostasis and ultimately system level AD pathogenesis.[4] Therefore, to investigate the foundational characteristics of these apoE lipoprotein particles it is necessary to purify a sufficient quantity of apoE lipoprotein particles in their native conformation for downstream analysis.

To accomplish this, we have developed a straightforward, adaptable, and scalable standardized immunoprecipitation (IP) protocol that uses the HJ15.4 monoclonal apoE antibody to isolate native apoE lipoprotein particles from multiple sources.[5–8] In this protocol, we first describe the culture conditions used to produce and collect the native apoE species in the immortalized astrocyte conditioned media. We then provide stepwise instructions for antibody coupling to the cyanogen bromide (CNBr)-activated Sepharose beads and sample incubation conditions. Lastly, we describe washing and elution conditions highlighting stopping points and considerations depending on

the downstream characterization assays of interest, including size exclusion chromatography (SEC), native PAGE, western blot, and lipidomics.

The methodology below was optimized and validated using native apoE lipoprotein particles from *APOE3/3* immortalized astrocyte conditioned media. However, this protocol has also been applied to iPSC-derived astrocyte conditioned media, iPSC-derived organoid conditioned media, human plasma, human cerebrospinal fluid, and postmortem human brain buffer-soluble lysate.

General experimental considerations and preparing reagents

Timing: ~ 2 h

1. Prepare all listed buffers and reagents before starting the protocol. Be sure that the pH is as listed for optimal efficacy.

   Note: It is recommended to make large stocks of these buffers and reagents for large-scale studies to reduce the technical variations between experiments.

2. Consider the apoE concentration of the input sample to determine the amount of HJ15.4 antibody conjugated beads that will provide optimal pull-down efficiency or sufficient apoE for the downstream assay of interest.

   Note: For example, we have observed that 25 μL of HJ15.4 conjugated beads (1 μg antibody/μL) saturated with 1 mL of 20× *APOE3/3* immortalized astrocyte conditioned media will pull-down and elute approximately 7 μg/mL of native apoE per quantification western blot, which can then be effectively characterized via SEC.

3. Consider the volume of the sample relative to the amount of HJ15.4 conjugated beads you plan to use. We recommend using a sample volume of at least 10× the bead volume to allow for equal distribution of the beads throughout the sample (i.e., 500 μL of sample for 50 μL of beads).
4. Determine the desired readout or purpose of the experiment, as there are multiple stopping points within the protocol depending on the experimental goal.

   Note: For example, to analyze the lipid composition of the isolated native apoE lipoprotein particles via lipidomics, the protocol can be terminated prior to the elution step.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
HJ15.4 mouse monoclonal anti-apoE antibody (1:1.28)	Liao et al.[7]	N/A
Mouse IgG Isotype Control antibody (1:3)	Invitrogen	Cat#10400C; Lot#XD345698; RRID: AB_2532980
WUE-4 Mouse monoclonal anti-apoE antibody (1:1000)	Abnova	Cat#MAB2371; RRID: AB_10548887
Goat anti-apoE polyclonal antibody (1:5000)	Meridian Life Science	Cat#K74180B; RRID: AB_150544
Streptavidin Poly-HRP40 conjugate antibody (1:2000)	Fitzgerald Industries International	Cat#50-125-3381
Chemicals, peptides, and recombinant proteins		
12 M hydrochloric acid	Fisher Chemical	Cat#A144-500
Sodium bicarbonate	Fisher Chemical	Cat#BP328-500
Sodium carbonate anhydrous	Fisher Chemical	Cat#S263-500

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
OmniPur® Sodium Chloride	Calbiochem	Cat#7760-5KG
5 M sodium chloride solution	Sigma-Aldrich	Cat#S6546-1L
Tris base	Fisher Bioreagents	Cat#BP152-5
Glacial acetic acid	Fisher Chemical	Cat#A38-212
Tween 20	Sigma-Aldrich	Cat#P7949
5% Sodium azide (w/v)	Ricca Chemical Company	Cat#7144.8-16
Sodium acetate, anhydrous	Sigma-Aldrich	Cat#S2889-1KG
Sodium phosphate	Sigma-Aldrich	Cat#342483-500G
Sodium thiocyanate	Fisher Chemical	Cat#S441-500
Sulfuric acid	Fisher Chemical	Cat#A300-500
Difco™ Skim Milk	BD Life Sciences	Cat#232100
PhosSTOP Phosphatase Inhibitor	Roche	Cat#4906837001
cOmplete Protease Inhibitor Cocktail	Roche	Cat#11836145001
UltraPure™ 0.5 M EDTA, pH 8.0	Invitrogen	Cat# 15575020
3,3′,5,5′-Tetramethylbenzidine Liquid Substrate, Super Slow	Sigma-Aldrich	Cat#T5569-100ML
DMEM/F-12, HEPES	Gibco	Cat#11330032
HyClone™ Fetal Bovine Serum	Cytiva	Cat#SH30396.03
100 mM sodium pyruvate	Gibco	Cat#11360070
Amphotericin B (Fungizone)	Gemini Bio Products	Cat#400-104
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Cat#15140-122
TrypLE™ Express Enzyme (1×), no phenol red	Gibco	Cat#12604013
Purified recombinant Human ApoE3 protein	Fitzgerald Industries International	Cat#30R2381
Critical commercial assays		
Pierce™ Coomassie (Bradford) Protein Assay Kit	Thermo Scientific	Cat#23200
Experimental models: Cell lines		
APOE3/3 immortalized Astrocytes	Morikawa et al.[6]	N/A
Software and algorithms		
UNICORN™ 7 WrkStn pure-BP-exp	Cytiva	Cat#29702890
Synergy H1 BioTek Gen 5 Software	Agilent	https://www.biotek.com/products/software-robotics-software/gen5-microplate-reader-and-imager-software/
Other		
GenClone TC Treated Flasks, Vent Growth Area: 75.0 cm^2	Genesee Scientific	Cat#25-209
GenClone TC Treated Flasks, Vent Growth Area: 182.0 cm^2	Genesee Scientific	Cat#25-211
CNBr-Activated Sepharose 4 Fast Flow	Cytiva	Cat#17098101
1.5 mL LoBind Microcentrifuge tubes	Eppendorf	Cat#022431081
Eppendorf® Microcentrifuge	Eppendorf	Cat#5424R
Sorvall™ Legend™ X1 Centrifuge	Thermo Fisher Scientific	Cat#75004261
ELMI RM-2L Intelli-mixer Large	Elmi	N/A
Fisherbrand™ accumet™ Liquid-Filled Mercury-Free pH/ATC Epoxy Body Combination Electrodes	Thermo Fisher Scientific	Cat#13-620-631
2 mL internal threaded polypropylene cryogenic vial	Corning	Cat#430488
Amicon® Ultra-15 Centrifugal Filter Unit, 10 kDa cutoff	Millipore	Cat#UFC901024
BD™ Luer Slip Tip Syringe sterile, single use	BD Syringe™	Cat#309659
26 G × 5/8 subcutaneous needles	BD Medical	Cat#305115
ÄKTA pure 25 L1	Cytiva	Cat#29018225
Superose® 6 Increase 10/300 GL	Cytiva	Cat#29-0915-96
ÄKTA pure 25 L1 Fraction Collector F9-C	Cytiva	Cat# 29027743
23 G 0.5″ Length Sterile Blunt Needles	SAI Infusion Technologies	Cat#B23-50
UNIPLATE Collection and Analysis Microplates	Cytiva	Cat# 7701-5200
Clear Flat-Bottom Immuno Nonsterile 96-Well Plates	Thermo Scientific	Cat# 439454
Synergy H1 Multimode Reader	BioTek	N/A

MATERIALS AND EQUIPMENT

Immortalized Astrocyte Growth Media		
Reagent	Final concentration	Add to 500 mL
Fetal Bovine Serum	10% (v/v)	50 mL
100 mM Sodium Pyruvate	1% (v/v)	5 mL
Penicillin-Streptomycin (10,000 U/mL)	1% (v/v)	5 mL
Amphotericin B (Fungizone)	1% (v/v)	5 mL
DMEM/F-12, HEPES	N/A	435 mL

Prepare media using aseptic techniques. Store at 4°C for up to two months. Protect from light. Before use, warm media to 37°C using a water or bead bath.

Immortalized Astrocyte Condition Media		
Reagent	Final concentration	Add to 500 mL
Fetal Bovine Serum	2% (v/v)	10 mL
DMEM/F-12, HEPES	N/A	490 mL

Prepare media using aseptic techniques. Store at 4°C for up to two months. Protect from light. Before use, warm media to 37°C using a water or bead bath.

Note: Depending on the study goals and considerations, immortalized astrocytes can be conditioned using serum-free DMEM/F-12, HEPES media. In this protocol, we decided to add 2% supplemental FBS to our condition media to keep the astrocytes in a more homeostatic state as it remains to be elucidated if astrocyte activation induces apoE lipoprotein particle changes in serum-free culturing condition.[9]

- 1× PhosSTOP Phosphatase Inhibitor: Dissolve 1 tablet per 10 mL of conditioned media.

10× stock solution can be made by adding 1 tablet to 1 mL of sterile water. Store at 4°C for up to one month or −20°C for at least 6 months.

- 1× cOmplete Protease Inhibitor Cocktail: Dissolve 1 tablet per 50 mL of conditioned media.

25× stock solution can be made by adding 1 tablet to 2 mL of sterile water. Store at 4°C for up to 2 weeks or −20°C for at least 3 months.

1 mM HCl pH 3.0		
Reagent	Final concentration	Add to 1 L
12 M HCl	1 mM	83.3 µL
Milli-Q H_2O	N/A	999.92 mL

Make sure pH is 3.0. Make small adjustments using NaOH and HCl as needed. Store solution at 20°C–22°C for up to one month. Avoid storage conditions that can cause vaporization (i.e., increased temperature or decreased pressure), as vaporization of HCl can cause decreased solution strength.

⚠ CRITICAL: Hydrochloric acid is corrosive and may cause irritation by all exposure routes. Make the above reagent under a chemical fume hood and wear proper protective gloves, clothing, and eye protection while handling.

Coupling Buffer pH 8.3		
Reagent	Final concentration	Add to 1 L
$NaHCO_3$	0.1 M	8.4 g
NaCl	0.5 M	29.22 g
Milli-Q H_2O	N/A	Bring to 1 L
Make sure pH is 8.3. Make small adjustments using NaOH and HCl as needed. Store at 20°C–22°C for up to 18 months.		

Note: It is recommended to add 800 mL of Milli-Q H_2O to the above reagents within a beaker and achieve the desired pH before bringing the total volume to 1 L. Mix utilizing a magnetic stir bar for at least five minutes.

0.1 M Tris-HCl Buffer pH 8.0		
Reagent	Final concentration	Add to 1 L
Tris Base	0.1 M	12.11 g
12 M HCl	0.1 M	8.35 mL
Milli-Q H_2O	N/A	Bring to 1 L
Make sure pH is 8.0. Make small adjustments using NaOH and HCl as needed. Store solution at 20°C–22°C for up to two weeks.		

0.1 M Tris-HCl with 0.5 M NaCl buffer pH 8.0

Reagent A: 0.1 M Tris Base with 0.5 M NaCl Buffer		
Reagent	Final concentration	Add to 1 L
Tris Base	0.1 M	12.11 g
NaCl	0.5 M	29.22 g
Milli-Q H_2O	N/A	Bring to 1 L

Reagent B: 0.1 M HCl with 0.5 M NaCl Buffer		
Reagent	Final concentration	Add to 1 L
12 M HCl	0.1 M	8.35 mL
NaCl	0.5 M	29.22 g
Milli-Q H_2O	N/A	Bring to 1 L
Make sure pH is 8.0 after adding Reagent A and Reagent B. Make small adjustments using NaOH and HCl as needed. Store solution at 20°C–22°C for up to two weeks.		

Note: To make the 0.1 M Tris-HCl with 0.5 M NaCl pH 8.0 working solution, add approximately 650 mL of Reagent B to 1 L of Reagent A and mix for at least five minutes using a magnetic stir bar.

0.1 M acetate with 0.5 M NaCl buffer pH 4.0

Reagent A: 0.1 M acetic acid with 0.5 M NaCl Buffer		
Reagent	Final concentration	Add to 1 L
Glacial Acetic Acid	0.1 M	5.75 mL
NaCl	0.5 M	29.22 g
Milli-Q H_2O	N/A	Bring to 1 L

Reagent B: 0.1 M sodium acetate with 0.5 M NaCl Buffer		
Reagent	Final concentration	Add to 1 L
Sodium Acetate	0.1 M	4.1 g
NaCl	0.5 M	14.6 g
Milli-Q H_2O	N/A	Bring to 500 mL
Make sure pH is 4.0 after adding Reagent A and Reagent B. Make small adjustments using NaOH and HCl as needed. Store solution at 20°C–22°C for up to two months.		

Note: To make the 0.1 M Acetate with 0.5 M NaCl working solution, add approximately 380 mL of Reagent B to 1 L of Reagent A and mix for at least five minutes using a magnetic stir bar.

- 0.02% Sodium Azide (NaN_3)-PBS: Add 400 µL of 5% Sodium Azide (w/v) stock to 99.6 mL of PBS.

Able to be stored at 20°C–22°C indefinitely.

⚠ CRITICAL: Sodium Azide is acutely toxic, corrosive, and both a health and environmental hazard. Prepare the above reagent within a chemical fume hood while equipped with proper protective equipment. Dispose of the above reagent and contaminated objects according to local, state, federal, and international regulations.

Wash Buffer 0.5 M NaCl in 20 mM Na_3PO_4		
Reagent	Final concentration	Add to 1 L
Sodium Phosphate (Na_3PO_4)	20 mM	3.278 g
NaCl	0.5 M	26.22 g
Milli-Q H_2O	N/A	Bring to 1 L
Store solution at 20°C–22°C indefinitely.		

Alternatives: Varying concentrations of NaCl ranging from 0.1 M to 0.5 M have been used depending on the study goal. In general, higher NaCl concentration may cause greater loss of apoE-associated proteins during the wash (step 25).

Elution Reagent 3 M NaSCN		
Reagent	Final concentration	Add to 1 L
Sodium Thiocyanate (NaSCN)	3 M	3.278 g
Milli-Q H_2O	N/A	Bring to 1 L
Store solution at 20°C–22°C indefinitely.		

⚠ CRITICAL: Sodium Thiocyanate is a health hazard and may cause damage to organs through prolonged exposure via ingestion, inhalation, or skin contact. When preparing the above reagent, work in a chemical fume hood with proper protective equipment.

SEC running buffer: 50 mM sodium phosphate buffer with 150 mM NaCl, 1 mM EDTA pH 7.4

Reagent A: 250 mM NaH_2PO_4 (monobasic)		
Reagent	Final concentration	Add to 200 mL
NaH_2PO_4	250 mM	6 g
Milli-Q H_2O	N/A	Bring to 200 mL

Reagent B: 250 mM Na_2HPO_4 (dibasic)		
Reagent	Final concentration	Add to 500 mL
Na_2HPO_4	250 mM	22.25 g
Milli-Q H_2O	N/A	Bring to 500 mL

Make sure pH is 7.4 after adding Reagent A and Reagent B. Make small adjustments using NaOH and HCl as needed. Remove gas from the final solution via vacuum filtration. Store at 4°C for up to one month.

Note: To make the SEC running buffer, add 800 mL of Milli-Q H_2O, 11.3 mL of Reagent A, 38.7 mL of Reagent B, 2 mL of 500 mM EDTA (Invitrogen), and 30 mL of 5 M NaCl (Sigma-Aldrich). Following pH adjustment, bring the solution to 1 L using Milli-Q H_2O, mix with a magnetic stir bar, and perform vacuum filtration of the solution to prevent damage to the Superose® 6 Increase 10/300 GL column during SEC.

WUE-4 ELISA 50 mM Carbonate Coating Buffer pH 9.6		
Reagent	Final concentration	Add to 1 L
Sodium Bicarbonate ($NaHCO_3$)	35 mM	2.93 g
Sodium Carbonate (Na_2CO_3)	16 mM	1.70 g
Sodium Azide (NaN_3)	0.02% (w/v)	0.2 g
Milli-Q H_2O	N/A	Bring to 1 L

Make sure pH is 9.6. Make small adjustments using NaOH and HCl as needed. Store solution at 20°C–22°C for up to three months.

- WUE-4 ELISA Blocking Buffer (1% Milk-PBS): 5 g of powdered skim milk in 495 mL of 1× PBS. Store solution at 4°C for up to one week.
- WUE-4 ELISA Washing Buffer (0.05% PBS-T): 500 µL of Tween 20 in 1 L of 1× PBS. Store solution at 20°C–22°C for up to three years.
- WUE-4 ELISA Stop Solution (1 M H_2SO_4): 28 mL of sulfuric acid in 472 mL of Milli-Q water. Store solution at 20°C–22°C indefinitely. Avoid storage conditions that can cause vaporization (i.e., increased temperature or decreased pressure), as vaporization of H_2SO_4 can cause decreased solution strength.

⚠ CRITICAL: Sulfuric acid is corrosive and can cause severe burns through skin contact, inhalation, and other exposure routes. Reaction with water is exothermic and should be performed within a chemical fume hood wearing proper protective equipment.

STEP-BY-STEP METHOD DETAILS

Immortalized astrocyte culture and condition media collection

Timing: ~ 1.5 weeks

Immortalized astrocytes are thawed and grown to confluency in 182 cm^2 culture flasks. Condition media is added, and after 72 h the native apoE particle-containing media is collected for subsequent IP.

Note: This protocol details the pull-down of native apoE lipoprotein particles from *APOE3/3* immortalized astrocyte conditioned media as the apoE particle source and will describe the workflow to characterization via SEC with supporting data. However, this protocol has been successfully applied to multiple model sources, including iPSC-derived astrocytes and iPSC-derived organoids, and human biospecimens, including postmortem buffer-soluble human brain lysate, human plasma, and human cerebrospinal fluid. Details regarding the application of this protocol to the above listed apoE lipoprotein particle sources can be

addressed through contacting the corresponding author. Therefore, this pull-down to characterization workflow can be adapted to the apoE source of interest and the desired characterization assay. Throughout the protocol, stopping points for specific downstream characterization techniques such as western blot, native PAGE, lipidomics, and SEC will be highlighted.

Note: It is recommended that a minimum of three biological replicates, specifically independent cultures with subsequent independent pull-down experiments, are performed to account for variation in apoE quantity, pull-down efficiency, and elution efficiency.

Note: During growth and conditioning of the immortalized astrocytes, we recommend preparing the stock of HJ15.4 antibody and mouse IgG isotype control conjugated beads for use during the subsequent IP.

1. Day 1. Thaw 2.0 × 10^6 cells stored in 10% DMSO-FBS solution at 37°C using a water or bead bath.
   a. Collect the cells and wash them in immortalized astrocyte growth media.
   b. Centrifuge the mixture at 300 g for 3 min.
   c. Aspirate the supernatant and resuspend the cells in 10 mL of fresh immortalized astrocyte growth media.
   d. Add them to a 75 cm^2 growth area flask for incubation.

Note: The immortalized astrocytes tend to clump together. Thus, to ensure an equal dispersion of cells be sure to resuspend the cells with moderate rigor and check for clumping using a light microscope before incubating the cells.

Note: 2.0 × 10^6 cells will typically reach confluency in a 75 cm^2 flask within 3 days of plating. If the cells have not reached confluency in this timeframe, continue to change the growth media every 3 days until confluency is reached.

2. Day 4. Once confluency is reached, aspirate the growth media, and add 5 mL of TrypLE Express Enzyme to dissociate the cells from the flask.
   a. Add 8 mL of growth media to the flask resuspending any remaining cells.
   b. Collect and centrifuge the mixture at 300 g for 3 min.
   c. Aspirate the supernatant and resuspend the cell pellet in 20 mL of growth media.
   d. Add resuspended cells to a 182 cm^2 growth area flask for incubation.

Note: The time to reach confluency will depend on the passage dilution. A 1:3 passage dilution will typically take three days to reach confluency in a 182 cm^2 growth area flask, but variation in the timeline has been observed.

3. Day 7. Once optimal confluency is reached, aspirate the growth media, and add 20 mL of immortalized astrocyte condition media (Figure 1).
   a. Incubate for 72 h before collecting apoE lipoprotein particle containing conditioned media.
4. Day 10. Collect the 20 mL of conditioned media.
   a. Centrifuge at 1,000 g for 5 min to remove cell debris.
   b. Transfer the supernatant to a separate 50 mL conical tube careful to not disrupt the pellet.
   c. Immediately add 1× PhosSTOP Phosphatase Inhibitor and 1× cOmplete Protease Inhibitor Cocktail.

Pause point: Store the condition media at 4°C for use within 1 week of collection or in −80°C for use within 1 month of collection.

Note: The storage conditions described above are based on the data we collected within the timelines of our optimization experiments. There was no significant difference in apoE

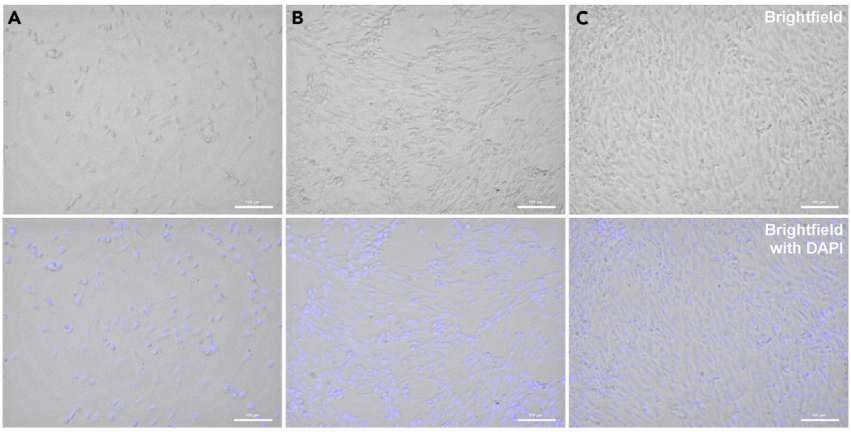


Figure 1. Representative images of *APOE3/3* immortalized astrocyte growth stages prior, during, and after optimal confluency for conditioning

APOE3/3 immortalized astrocytes were seeded at a density of 2.5 × 10^5 cells in 9.6 cm^2 growth area plates and cultured according to the above-described conditions (A-C).

The cells were fixed with 4% paraformaldehyde (PFA) and stained with 4′,6-diamidino-2-phenylindole (DAPI) prior to optimal conditioning cell confluency (A), during optimal conditioning confluency (B), and after optimal conditioning confluency (C). Brightfield and DAPI images were taken at 20× magnification. Scale bar = 100 μm.

quantity (Fresh = 2.9 ± 0.453 μg/mL; Frozen = 2.4 ± 0.504 μg/mL; p = 0.28) or apoE lipoprotein particle size distribution following one −80°C freeze-thaw event (Figure 2). The effect of multiple freeze-thaw events on native apoE lipoprotein particles was not thoroughly examined; thus, we recommend performing the IP using fresh media or following one freeze-thaw cycle.

HJ15.4 and mouse IgG isotype control antibody bead coupling- day 1

Timing: 5–6 h

HJ15.4 anti-apoE antibody and mouse IgG isotype control antibody are coupled to CNBr-Activated Sepharose 4 Fast Flow beads for pull-down of native apoE lipoprotein particles.

Alternatives: HJ15.4 is a mouse monoclonal anti-apoE antibody, which has been validated to effectively pull-down native apoE lipoprotein particles per the described protocol. However, there are other alternative mouse monoclonal anti-apoE antibodies commercially available including WU-E4; however, we have not validated that these alternative antibodies pull-down native lipidated apoE lipoprotein particles.

5. Weigh a pre-determined amount of CNBr-Activated Sepharose 4 Fast Flow beads for desired bead stock volume.
   a. Add to a 50 mL conical tube.

   Note: 1 g of beads will swell to approximately 3 mL of beads. The volumes described below will assume 1 g of beads are used, but these volumes can be adjusted proportionally for the desired scale.

6. Add 20 mL of 1 mM HCl to 1 g of beads creating a homogeneous mixture.
   a. Incubate the beads with mild agitation using program F8 (13 rpm) of the ELMI RM-2L Intellimixer for 45 min at 20°C–22°C.
   b. Centrifuge the mixture at 300 g for 1 min.

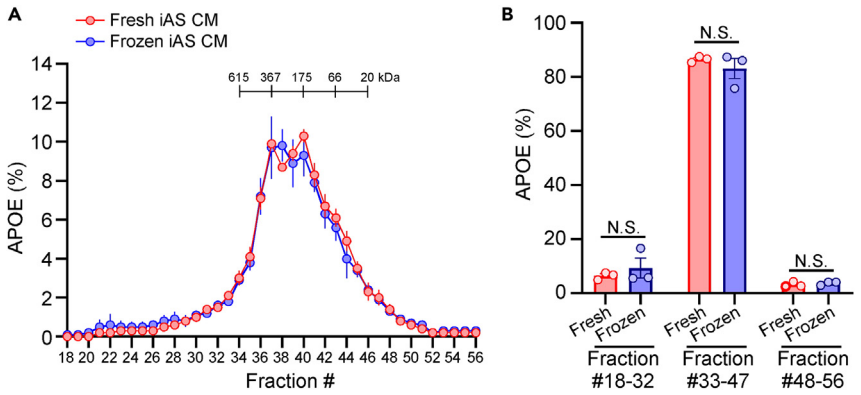


Figure 2. ApoE size distribution of fresh and frozen *APOE3/3* immortalized astrocyte conditioned media (iAS CM)

Three independent *APOE3/3* immortalized astrocyte batches were grown to confluency in 182.0 cm^2 growth flasks and conditioned in 20 mL of 2%-FBS containing media for 72 h prior to collection. The media was split into two 10 mL aliquots, one of which was immediately frozen at −80°C, and the other was utilized for SEC. After 48 h, the frozen sample was thawed at 4°C and utilized for SEC. WUE-4 ELISA was performed using fractions 18–56 for both samples, and the size was calculated using commercial protein standard (A). The fractions were then divided into tertiles defined by size (≥615 kDa–20 kDa≥). The area under the curve (AUC) was calculated and paired t-tests (two-tailed) were performed utilizing GraphPad Prism 9.4.1. Data are represented as mean ± SEM. $P < 0.05$ is considered statistically significant. N.S. = Not significant (B).

c. Aspirate the supernatant.

7. Wash the beads by adding 20 mL of Coupling buffer.
   a. Centrifuge at 300 g for 1 min and aspirate the supernatant.
8. Resuspend the beads in Coupling buffer with enough volume to create a 1:1 slurry (i.e., 3 mL of beads + 3 mL of Coupling buffer produces 6 mL slurry).
   a. Transfer the slurry to a 15 mL conical tube for antibody coupling.
9. Dilute the stock antibody solution to 1 mg/mL using coupling buffer as the diluent with equal volume to the bead volume (i.e., 3 mL of 1 mg/mL antibody per 3 mL of beads).

⚠ CRITICAL: To ensure that effective antibody bead coupling has occurred, a Bradford Protein Assay will be performed comparing the diluted antibody input to the remaining antibody in the supernatant following the coupling reaction. Be sure to save at least 5 µL of the diluted antibody input for this purpose.

10. Aspirate the supernatant and add the diluted antibody solution to the swelled beads.
    a. Allow the coupling reaction to occur at 20°C–22°C with end-over-end mixing (program F1, 13 rpm) for 2.5 h.
11. Briefly centrifuge the mixture at 300 g for 1 min.
12. Remove 5 µL of the supernatant and perform a Bradford Coomassie protein assay to determine coupling efficacy (Figure 3).

Note: If a significant amount of protein remains in the supernatant following the initial coupling reaction, continue the reaction for at least another 30 min before repeating measurements.

13. Aspirate the remaining supernatant carefully to minimize bead loss.
    a. Wash the beads by adding 2 bead volumes of coupling buffer.
    b. Centrifuge at 300 g for 1 min.
    c. Aspirate the supernatant.
14. Quench the unreacted groups on the beads by resuspending with 1 bead volume of 0.1 M Tris-HCl pH 8.0.

A

	BSA Standard		Pre-Coupling Antibody Input		Post-Coupling Supernatant		
	D1	D2	D1	D2	D1	D2	
	1	2	3	4	5	6	
A	1998.7	2003.3	613.1	651.4	17.2	25.2	S1
B	1000.1	993.9	676.3	605.2	28.8	51.6	S2
C	495.5	512.4	597.6	628.0	26.2	14.3	S3
D	254.3	244.9					
E	122.1	114.7					
F	59.7	83.7					
G	25.7	28.6					
H	<0.000	1.2					

B

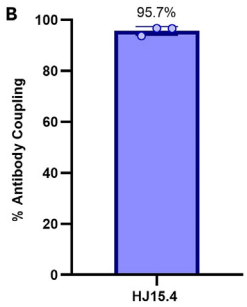


Figure 3. Representative Bradford Coomassie protein assay results for verification of HJ15.4 antibody coupling to CNBr-Activated Sepharose 4 Fast Flow beads

Three independent antibody coupling reactions were performed for 2.5 h at 20°C–22°C as described in the protocol above. A bovine serum albumin (BSA) standard curve was generated via serial dilution (2000 ng/mL- 0 ng/mL) and 5 µL of the pre-coupling diluted HJ15.4 antibody and the post-coupling supernatant was added to the indicated wells in duplicate, respectively (A). All samples were incubated with 150 µL of Coomassie reagent for 10 min at 20°C–22°C prior to measuring absorbance at 595 nm on a plate reader. Lastly, the mean protein concentration (ng/mL) remaining in the supernatant was divided by the mean protein concentration in the pre-coupling input to determine the percentage of antibody coupled to the beads. Data are represented as mean ± SEM (B). D1-2, Duplicate 1 and Duplicate 2; S1-3, Sample 1–3.

a. Perform end-over-end mixing (program F1, 13 rpm) for 2 h at 20°C–22°C.

Alternatives: Quenching of unreacted groups can also be accomplished utilizing 1 M ethanolamine pH 8.0.

15. Wash the beads by alternating between 1 bead volume of 0.1 M Tris-HCl pH 8.0 with 0.5 M NaCl and 1 bead volume of 0.1 M Acetate pH 4.0 with 0.5 M NaCl for three consecutive rounds.
    a. Between each resuspension, centrifuge at 300 g for 30 s to pellet the beads and aspirate the supernatant.
16. Following the third round of washing, aspirate the supernatant and wash the antibody conjugated beads with 1× PBS for use in IP.

Pause point: The antibody conjugated beads can be used immediately for IP or can be stored in at least two bead volumes of 0.02% NaN_3-PBS at 4°C for prolonged periods of time.

Sample concentration and incubation- day 1

Timing: 2 h (with ~ 16h incubation period)

APOE3/3 immortalized astrocyte conditioned media is concentrated to the desired apoE concentration. The sample is then added to HJ15.4 and mouse IgG isotype control conjugated beads for native apoE lipoprotein particle pull-down.

Note: The below steps will assume the conditioned media has been frozen at −80°C following step 4, and a large stock of antibody conjugated beads was stored in 0.02% NaN_3-PBS at 4°C following step 16.

17. Thaw the immortalized astrocyte conditioned media at 4°C or on ice.

Note: Conditioned media takes multiple hours to thaw at 4°C. We recommend that the media is thawed early in the day to allow ample time. We also recommend preparing the beads for sample incubation during this waiting period (step 20).

18. Centrifuge the conditioned media at 1,000 g for 5 min.
    a. Transfer the supernatant to a separate 50 mL conical tube careful to not disrupt the pellet.
19. Concentrate the conditioned media to the desired apoE concentration by adding 15 mL of conditioned media to one Amicon® Ultra-15 Centrifugal Filter Unit.
    a. Centrifuge at 4,500 g for 20 min at 4°C.
    b. Remove 5 mL of the flow-through and add the remaining 5 mL of conditioned media.
    c. Centrifuge at 4,500 g for 15 min at 4°C, or until the conditioned media has reached the desired volume (i.e., for a 20× concentration continue centrifugation until 1 mL of concentrated media remains).
    d. Collect the media from the centrifugal filter unit and store it in LoBind microcentrifuge tubes on ice.

Note: The input apoE concentration should be determined based on the desired experimental outcome. For example, if the goal is to profile the size of apoE lipoprotein particles via SEC, a maximal possible input concentration is advised to maximize purified apoE quantity.

Note: Based on our experience with our *APOE3/3* immortalized astrocytes, we have observed that 1× conditioned media contains approximately 2.5 μg/mL of apoE per WUE-4 ELISA.

20. Acquire the previously prepared HJ15.4 and IgG conjugated beads for washing.
    a. Centrifuge at 300 g for 1 min to pellet the beads.
    b. Remove 0.02% NaN_3-PBS to 1 bead volume. Resuspend the beads to create a 1:1 bead slurry.
    c. Transfer the desired amount of bead slurry to LoBind microcentrifuge tubes.

Note: We recommend that the sample volume is at least 10× the bead volume for equal distribution of beads throughout the media (i.e., 500 μL of sample for 50 μL of beads).

21. Wash the beads with 1× PBS in excess to remove the residual 0.02% NaN_3.
22. Centrifuge at 300 g for 1 min to pellet the beads and aspirate the wash buffer.

⚠ CRITICAL: Be careful to minimize bead loss in this step. The HJ15.4 and IgG isotype control beads should be equal in volume to have a proper negative control.

23. Add the immortalized astrocyte conditioned media to the antibody conjugated beads.
    a. Incubate the samples approximately 16 h at 4°C with end-over-end mixing and mild agitation (program F4, 13 rpm).

Bead wash and native ApoE lipoprotein particle elution- day 2

Timing: 2 h

The native apoE-bound beads are washed to remove nonspecific proteins, and the particles are eluted for downstream characterization.

Note: Stopping points for lipidomics will be pointed out, and workflows towards western blot and native PAGE will also be noted in addition to the primary description of SEC.

⚠ CRITICAL: Minimization of bead loss during the following steps is critical to maximize the quantity of purified apoE lipoprotein particles collected. Therefore, when collecting the supernatant, washing buffer, and eluent use syringes and 26 G × 5/8 subcutaneous needles with the bevel of the needle facing the wall of the LoBind microcentrifuge tube. We

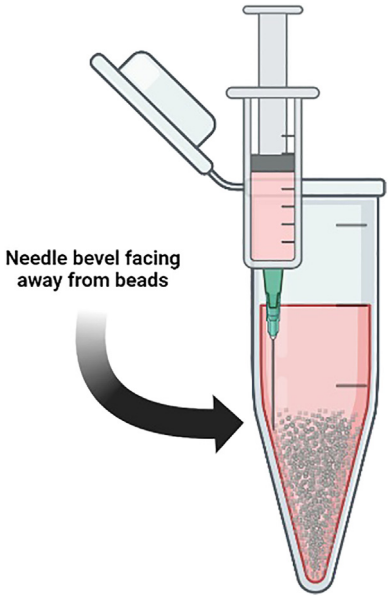


Figure 4. Syringe and needle sample collection technique for minimization of bead loss

recommend placing the bevel of the needle where the beads and supernatant intersect to prevent puncturing the bottom of the tube (Figure 4).

24. Centrifuge the apoE-bound beads at 300 g and 4°C for 1 min to pellet the beads.
    a. Aspirate the supernatant using the above-described syringe and needle technique.

Note: To measure pull-down efficiency via western blot, this supernatant can be saved and stored at 4°C.

25. Resuspend the beads in at least 3 bead volumes of 0.5 M NaCl wash buffer.
    a. Centrifuge at 300 g and 4°C for 1 min to pellet the beads.
    b. Aspirate the wash buffer using the syringe and needle technique.

Note: For lipidomics, the beads can be resuspended in 1 bead volume of 1× PBS and stored at –80°C following this step. The samples can then be eluted and analyzed according to previously described protocols.[10,11]

26. Resuspend the beads in at least 4 bead volumes of 3 M NaSCN elution reagent.
    a. Incubate at 20°C–22°C for at least 1.5 h with end-over-end mixing and mild agitation (program F4, 13 rpm).
27. Centrifuge at 300 g and 4°C for 1 min to pellet the beads.
    a. Collect the native apoE lipoprotein particle containing elution reagent using the syringe and needle technique.

Pause point: Store the native apoE lipoprotein particles at 4°C while preparing for SEC.

Note: To measure the elution efficiency via western blot, the beads can be resuspended in 1× SDS containing sample buffer and heated at 95°C for 10 min to elute any remaining apoE (Figure 5). The elution reagent can be used directly for western blot and native PAGE. The apoE band may appear at a slightly higher molecular weight when imaging due to the high salt content in the elution reagent.

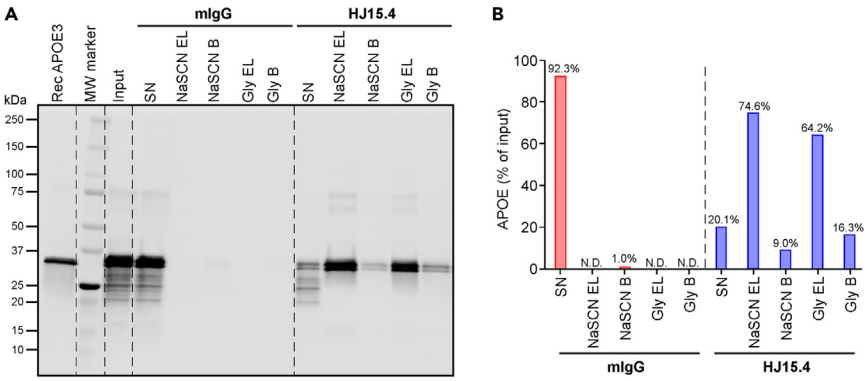


Figure 5. HJ15.4 relative apoE pull-down and elution efficiency

APOE3/3 immortalized astrocytes were grown to confluency in two 182.0 cm^2 growth flask and conditioned in 20 mL of 2%-FBS containing media for 72 h prior to collection. The conditioned media was concentrated 20× and 1 mL of media (Input) was added to 50 µL of HJ15.4 monoclonal mouse anti-apoE antibody (HJ15.4) or the mouse IgG isotype control (mIgG) conjugated beads for IP, as described above. The beads were split into 25 µL aliquots following washing (step 25) and were eluted with 4 bead volumes of 3 M NaSCN (NaSCN EL) or 0.1 M glycine pH 2.5 (Gly EL). The eluent was collected, and the beads were resuspended in 1× SDS-containing sample buffer to elute any remaining apoE lipoprotein particles (NaSCN B and Gly B). A western blot was performed utilizing 1% of each respective sample, and K74180B (1:2000) was used for primary antibody detection (A). The membrane was imaged, and signal strength was quantified using Odyssey® CLx Infrared Imaging System by LI-COR Biosciences (B). Pull-down efficiency is estimated by calculating the percentage of apoE remaining in the supernatant sample (SN) post-16-h incubation compared to the input sample. Elution efficiency has been defined as the difference in apoE quantity pulled down and quantity of apoE eluted.

Note: 3 M NaSCN is used as the elution reagent in this protocol, instead of the commonly used 0.1 M glycine, because of concerns regarding apoE lipoprotein particle integrity utilizing pH-dependent elution. Previous studies have successfully utilized 3 M NaSCN for apoE lipoprotein particle elution while maintaining particle integrity.[5,6]

Size exclusion chromatography of purified ApoE lipoprotein particles- day 2

Timing: 4.5 h

The purified native apoE lipoprotein particles are diluted with SEC running buffer and manually injected into the ÄKTA pure 25 L1 FPLC system. The sample is run through tandem Superose® 6 Increase 10/300 GL columns fractionating the apoE lipoprotein particles and apoE-associated proteins by size for particle characterization.

Alternatives: There are multiple FPLC and HPLC systems commercially available such as BioRad NGC Quest Chromatography System and Agilent 1260 Infinity II Bio-SEC Multi-Detector System. These analogous systems and their associated columns utilize the same fundamental principles of SEC, thus should generate comparable data. If using an alternative FPLC or HPLC system, we recommend utilizing the below protocol as guidelines that can be adapted according to the specifics of the alternative system.

Note: Before beginning the below SEC protocol, make sure the ÄKTA pure 25 L1 FPLC system has been cleaned and prepared properly according to the ÄKTA pure user manual to avoid contamination from previous runs.

Note: Our system has been configured such that the SEC running buffer is attached to inlet A1. Additionally, for our protocol optimization experiments, we typically use a 0.5 mL capillary loop for manual injection. However, this can be scaled up as needed.

⚠ CRITICAL: The columns used for SEC are two Superose® 6 Increase 10/300 GL columns connected in tandem. The column volume is 23.562 mL with a pre-column pressure limit of 5.0 MPa and a ΔC pressure limit of 2.60 MPa. The flow rate for all steps is 0.3 mL/min. These columns are easily damaged by gases, so take precautions to not introduce gases into the FPLC system.

28. Dilute the apoE lipoprotein particle containing eluent with additional 3 M NaSCN to bring the total volume to the loop injection volume.
    a. Add an excess of SEC running buffer to prevent gases from being introduced into the system during the protocol execution.

Note: For example, using a 0.5 mL capillary loop and 100 μL eluent, add 400 μL of additional 3 M NaSCN elution reagent and at least 150 μL of SEC running buffer to bring the total sample volume to 650 μL.

29. Using a syringe and blunt tip needle carefully draw up the sample.
    a. Invert the syringe, such that the needle is above the syringe, and remove the blunt tip needle.
    b. Gently tap the syringe to remove gases from the sample prior to manual injection.

⚠ CRITICAL: Be sure all gas bubbles have been removed from the sample prior to injection into the FPLC system.

30. Inject the sample into the capillary loop via the injection port.
    a. Leave the syringe attached to the injection port throughout the protocol execution to prevent gases from being introduced into the system.

Note: The below steps detail the SEC protocol parameters that will be inputted into the UNICORN™ 7 WrkStn pure-BP-exp software to execute SEC. Once this protocol has been created the system will execute it automatically.

31. Equilibration.
    a. Flow rate: 0.3 mL/min.
    b. Inlet: A1.
    c. Equilibrate until total volume is 0.3 column volumes (CV).
    d. Reset UV monitor.
32. Sample Application.
    a. Flow Rate = 0.3 mL/min.
    b. Loop injection: manually load.
    c. Loop type: capillary loop.
    d. Empty loop: 0.50 mL (or chosen loop volume).
    e. Inlet: A1.
    f. No fractionation.
33. Elution.
    a. Flow rate = 0.3 mL/min.
    b. Inlet: A1.
    c. Isocratic elution volume 3.00 CV.
    d. Fractionation Type: Fixed volume fractionation.
    e. Fractionation destination: 96-deep well plate.
    f. Fixed fractionation volume: 0.80 mL.
    g. Stop Fractionation at the end of this phase.
34. Insert a 96-well UNIPLATE Collection and Analysis Microplate into the ÄKTA pure 25 L1 Fraction Collector F9-C.
    a. Start the protocol.

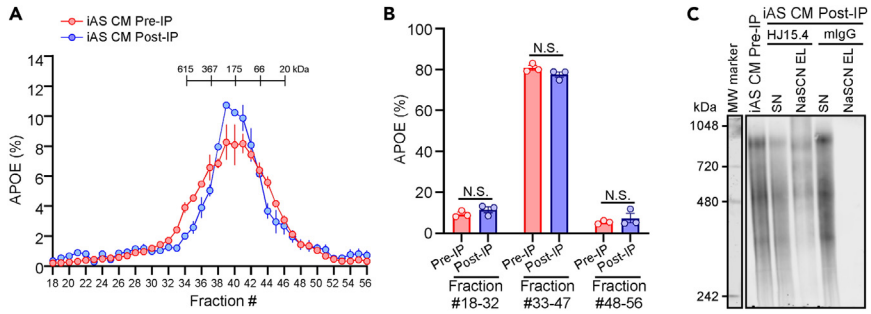


Figure 6. Comparison of apoE lipoprotein particle size distribution in *APOE3/3* immortalized astrocyte conditioned media (iAS CM Pre-IP) and purified apoE lipoprotein particles in elution reagent following HJ15.4 IP protocol (iAS CM Post-IP)

Three independent *APOE3/3* immortalized astrocyte batches were grown to confluency in 182.0 cm^2 flasks, and the apoE IP protocol was performed as described above. Following elution, SEC of both the pre-IP and the post-IP samples was performed, and WUE-4 ELISA was used to quantify apoE concentrations of the selected fractions (A). The fraction size was calculated using a commercially available protein standard and the fractions were then divided into tertiles defined by size (≥615 kDa–20 kDa≥). The area under the curve (AUC) of each tertile was calculated and paired t-tests (two-tailed) were performed utilizing GraphPad Prism 9.4.1 (B). Data are represented as mean ± SEM. $P < 0.05$ is considered statistically significant. N.S. = Not significant. Lastly, native PAGE detecting apoE (K74180B; 1:2000) was performed utilizing samples from a single pre-IP and post-IP batch for qualitative validation (C). *APOE3/3* immortalized astrocyte conditioned media, iAS CM Pre-IP; supernatant (step 24), SN; Eluted apoE lipoprotein particles, NaSCN EL.

Note: The above-described SEC protocol will take approximately 261 min to execute.

ApoE ELISA of fractionated native ApoE lipoprotein particles

Timing: 3 days

The size distribution of purified native apoE lipoprotein particles is quantified using an apoE ELISA of selected SEC fractions. The apoE quantity per fraction is plotted as a percentage of the total apoE in the selected fractions (Figure 6A).

Note: Depending on the experimental goal, the anti-apoE capture antibody used may need optimization. In the below protocol we use the WUE-4 mouse monoclonal anti-apoE antibody, which we have observed to have isoform specificity. Therefore, it is recommended that WUE-4 is only used to compare between samples of the same *APOE* genotype.

Note: All samples added to the 96-well ELISA plate will be 100 μL in volume unless otherwise indicated.

35. Day 1: Coat Clear Flat-Bottom Immuno Nonsterile 96-Well Plates with WUE-4 antibody.
    a. Dilute 1 mg/mL WUE-4 antibody stock 1:1000 in ELISA coating buffer.
    b. Incubate ∼ 16h at 4°C.

Note: We recommend coating ELISA plates the day before performing SEC for optimal efficiency.

36. Day 2: WUE-4 ELISA plate blocking and sample incubation.
    a. Aspirate WUE-4 coating buffer.
    b. Block ELISA plates with 200 μL of 1% Milk-PBS for at least 3 h at 20°C–22°C.
    c. Prepare recombinant human ApoE3 standard, with appropriate replicates, ranging from 20,000 pg/mL to 0 pg/mL using blocking buffer as diluent.

d. Acquire the 96-well UNIPLATE Collection and Analysis Microplate containing the fractionated apoE sample.
e. Dilute fractions 18–56 (Wells 3B–7H) with blocking buffer as the diluent, such that the signal will fall within the standard curve.

Note: For 25 μL of apoE saturated beads and a 100 μL elution volume prior to SEC, we have repeatedly used a 1:10 dilution for the apoE fractions. However, identifying the proper dilution factor might need to be optimized depending on the experimental design. For accurate quantification, be sure the samples fall within the standard curve.

f. Aspirate the blocking buffer and wash the plates three times with 0.05% PBS-T.
g. Add diluted standard and sample. Incubate ∼ 16h at 4°C.

37. Day 3: WUE-4 ELISA Detection.
    a. Dilute K74180B antibody 1:5000 of 1 mg/mL stock in blocking buffer.
    b. Aspirate the sample and wash three times with 0.05% PBS-T.
    c. Add diluted detection antibody.
    d. Incubate for 2 h at 20°C–22°C.
    e. Dilute stock streptavidin Poly-HRP40 conjugate antibody 1:2000 in blocking buffer.
    f. Aspirate the detection antibody and wash the plates three times with 0.05% PBS-T.
    g. Add diluted streptavidin antibody.
    h. Incubate for 1 h.
    i. Aspirate and wash three times with 0.05% PBS-T.
    j. Add 3,3′,5,5′-Tetramethylbenzidine super slow liquid substrate to sample and incubate for approximately 1 min or until the colorimetric reaction starts to saturate.
    k. Add ELISA stop solution to stop the colorimetric reaction.
    l. Measure absorbance at 450 nm using the Synergy H1 Multimode Reader.
38. Convert the raw absorbance values to apoE concentration utilizing the standard curve.

Note: By taking the concentration of each individual fraction and dividing it by the sum of the total apoE concentration, a scatter plot can be produced to display the size distribution of the purified apoE lipoprotein particles (Figure 6A). The corresponding molecular weight for each fraction can be estimated by performing the described SEC protocol using protein standards.

EXPECTED OUTCOMES

A successful IP of native apoE lipoprotein particles from *APOE3/3* immortalized astrocyte conditioned media should result in an elution efficiency of approximately 95% and a pull-down efficiency of approximately 80%, depending on the apoE input concentration (Figure 5). When utilizing this protocol to pull down apoE lipoprotein particles from other apoE sources, such as buffer soluble human brain lysate or iPSC-derived cell line conditioned media, there will be variation in IP efficiency. Furthermore, the IP efficiency will vary depending on the quantity of apoE lipoprotein particles within the input sample in proportion to the quantity of HJ15.4 beads. The IP can be optimized to reach maximal pull-down efficiency; however, in most cases, sufficient apoE lipoprotein particle quantity can be reached via saturation of the HJ15.4 beads. For example, in apoE lipoprotein particle SEC characterization experiments, we have found that 25 μL of apoE saturated HJ15.4 beads results in the elution of approximately 7 μg/mL of apoE lipoprotein particles, which is sufficient for analysis via SEC, ELISA, western blot, native PAGE, and lipidomics.

The primary novel application of this protocol is to examine the characteristics of *native lipid-bound* apoE lipoprotein particles and utilize these native particles in downstream experiments. Therefore, validation that the above HJ15.4 IP protocol maintains apoE lipoprotein particle integrity and lipidation status is vital. To do so, we characterized the isolated apoE lipoprotein particles via SEC,

native PAGE, and lipidomics. Comparison of the apoE lipoprotein particle size distribution from pre-IP *APOE3/3* immortalized astrocyte conditioned media and post-IP purified apoE lipoprotein particles via SEC and native PAGE, shows no significant difference in the area under the curve (AUC) nor significant qualitative difference in size distribution (Figure 6). Taken together, this suggests that the isolated apoE lipoprotein particles remain intact following IP.

Furthermore, lipidomic analysis of the isolated apoE lipoprotein particles suggests that the particles remain lipidated and are particularly enriched with cholesterol (total cholesterol = 4,200 nmol/mg apoE; free cholesterol = 873 nmol/mg apoE), sphingomyelin (SM), phosphatidylcholine (PC), and phosphatidylethanolamine (PE). Interestingly, lysophosphatidylcholine (LPC) or lysophosphatidylethanolamine (LPE) were detected at similar levels between HJ15.4 and mIgG beads, suggesting that these lipids may not be present or are present at a minimal level in apoE lipoprotein particles secreted by immortalized astrocytes (Figure 7). In conjunction, these results suggest that the purified apoE lipoprotein particles remain in their native lipidated conformational state following the HJ15.4 IP protocol.

LIMITATIONS

Although the HJ15.4 IP has a high pull-down and elution efficiency when optimized, the protocol requires that the apoE lipoprotein particle concentration in the input sample is high enough so that it can be detected and used for downstream assays. This can pose a challenge when utilizing model systems such as iPSC-derived cell lines, particularly if the cell type does not produce high quantities of apoE physiologically, such as microglia and neurons. To solve this problem, the investigator could consider a large-scale cell culture to increase the input sample concentration. Furthermore, the issue of apoE lipoprotein particle concentration can be exacerbated by multiple freeze-thaw events, although we did not observe a significant change in apoE lipoprotein particle size or quantity after a single −80°C freeze-thaw event.

Another limitation of this protocol is the high salt content of the elution reagent, which can impact downstream characterization assays. The high salt content does not appear to affect western blot, native PAGE, silver stain, ELISA, or SEC. However, 3 M NaSCN does interfere with the Amplex Red cholesterol assay. If elution reagent interference is observed for the assay of interest, dialysis of the apoE lipoprotein particles into 1× PBS can be performed.

TROUBLESHOOTING

Problem 1

Low immortalized astrocyte cell viability or apoE lipoprotein particle production (step 3).

Potential solution

There are multiple possible explanations for low immortalized astrocyte cell viability and subsequent apoE lipoprotein particle production. To maximize cell viability first ensure cells were cryopreserved in appropriate cryopreservation agents, such as 10% DMSO-FBS, and are of a limited passage number to minimize the possibility of genetic drift, loss of astrocytic characteristics, and senescence. Additionally, avoid overgrowth of cells by carefully monitoring their growth and adjusting culture conditions as necessary, such as passaging to larger flasks or changing the seeding density of the cells. Lastly, be sure to add condition media at the appropriate confluence to maximize apoE lipoprotein particle concentration and minimize astrocyte activation.

Of note, *APOE* genotype can also influence the quantity of apoE lipoprotein particles produced. For example, we have observed that *APOE2/2* immortalized astrocytes produce increased apoE lipoprotein particle levels when compared to *APOE3/3* immortalized astrocytes while *APOE4/4* immortalized astrocytes produce the lowest quantity of the isoforms.

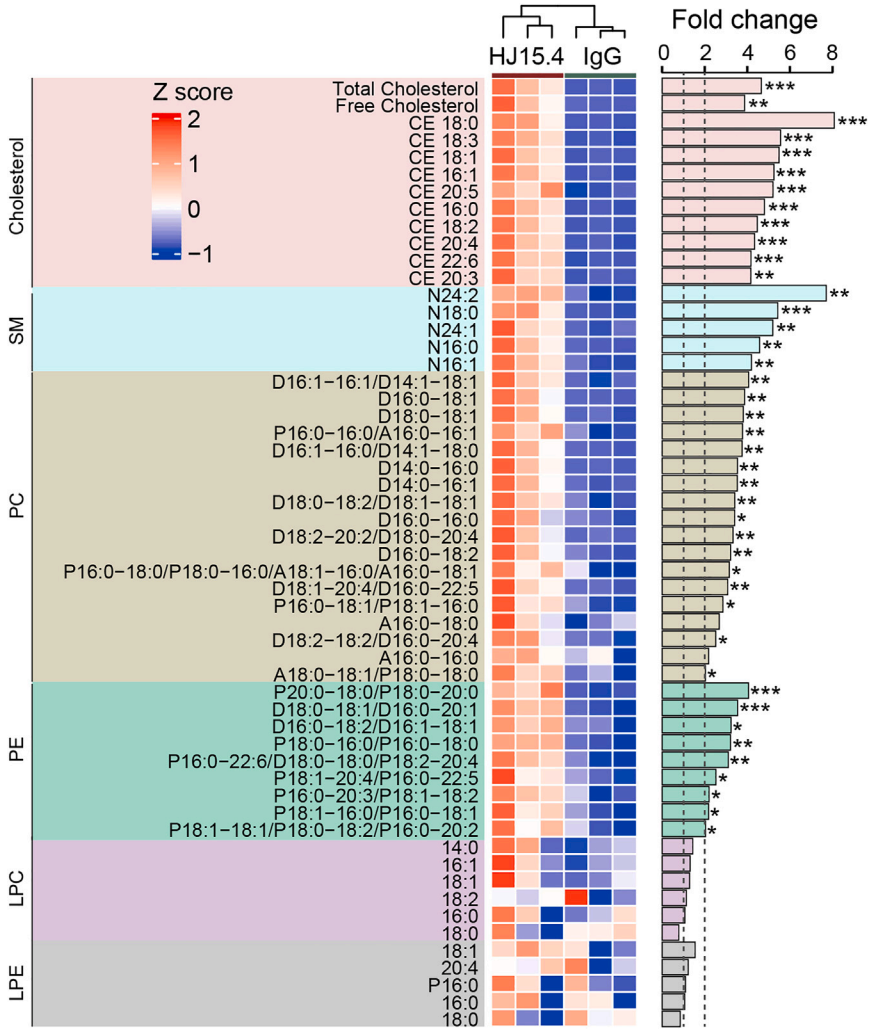


Figure 7. Lipids associated with apoE lipoprotein particles isolated from *APOE3/3* immortalized astrocyte conditioned medium

Heatmap (middle panel) shows scaled levels of 55 lipid species measured by mass spectrometry of six independent apoE isolation assays using the HJ15.4 monoclonal mouse anti-apoE antibody (HJ15.4) or the isotype mouse IgG control (IgG). The amount of each lipid species was compared between the HJ15.4 and IgG groups using linear models (right panel). The lipid species with a fold change ≥ 2 and P value < 0.05 are considered apoE lipoprotein particle-associated lipids. The lipids were normalized to volume of CNBr beads for the pull-down assay (25 μL). The fold change and P values comparing the lipid amount in the HJ15.4 and IgG groups were calculated using linear regression models. The analysis was done using R 4.1.2. CE, Cholesterol Ester; SM, Sphingomyelin; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; LPC, Lysophosphatidylcholine; LPE, Lysophosphatidylethanolamine. ***P < 0.001; **P < 0.01; *P < 0.05.

Problem 2

Low or inadequate HJ15.4 antibody coupling efficacy (step 12).

Potential solution

Adequate antibody coupling efficacy is essential for effective apoE IP and we have repeatedly measured a coupling efficacy of approximately 96% for HJ15.4 using the above-described conditions. If significant antibody remains in the supernatant of the coupling reaction, we recommend continuing the reaction for at least 30 min prior to remeasuring the antibody concentration using the Bradford Coomassie assay (thermofisher.com). Additionally, complete washing (steps 8 and 9)

as quickly as possible prior to performing the coupling reaction. Prolonged washing will lead to a reduction in the coupling efficiency.

Problem 3

Low pull-down efficiency (step 23).

Potential solution

We have found that apoE lipoprotein particle pull-down efficiency is highly variable depending on the apoE concentration of the input sample, which often varies from batch to batch depending on the model system. If the goal is to optimize for pull-down efficiency, we recommend performing a serial dilution of the input sample utilizing a constant volume of beads to determine the optimal input volume prior to performing critical experiments. However, for most cases of downstream characterization assays, we have found that saturating the beads with apoE lipoprotein particles to maximize the quantity of apoE pulled down has been sufficient irrespective of pull-down efficiency.

Problem 4

Low elution efficiency (step 26).

Potential solution

Depending on the model system or human biospecimen used as the apoE source, the elution efficiency might vary. We have observed a lower elution efficiency when using iPSC-derived organoid-conditioned media. In this protocol, the listed timeline was optimized for *APOE3/3* immortalized astrocyte conditioned media. For other sources of apoE, the investigators might need to increase the time for sample incubation and optimize the ratio between input sample and beads if a low elution efficiency is observed.

Problem 5

Anomalous apoE lipoprotein particle size distribution (step 38).

Potential solution

When performing SEC of lipoprotein particles, multiple factors can cause an anomalous size distribution that is not indicative of the native lipoprotein particles being produced by the model system or contained in the biospecimen. These factors include aggregation, degradation, and loss of lipids during the sample preparation prior to SEC. Therefore, we recommend performing SEC immediately following apoE lipoprotein particle isolation to avoid inaccurate size determination. Additionally, we recommend validating SEC results with complementary techniques such as native PAGE.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Na Zhao (zhao.na@mayo.edu).

Materials availability

HJ15.4 mouse monoclonal anti-apoE antibody and *APOE3/3* immortalized astrocytes are available from Dr. David M. Holtzman's laboratory, assuming requestor is willing to sign a material transfer agreement (MTA) with Washington University in St. Louis. All other materials, including alternative anti-apoE antibody WU-E4, are available for commercial purchase.

Data and code availability

This study did not generate or analyze any novel datasets or code.

ACKNOWLEDGMENTS

This work was supported by NIH grants U19AG069701 (to D.M.H., X.H., and N.Z.), RF1AG046205 (to N.Z.), and RF1AG061872 (to X.H.); a BrightFocus Foundation grant (to N.Z.); a BrightFocus Foundation fellowship (to A.-C.R.); and a Mayo Clinic Alzheimer's Disease Research Center Developmental Grant (to N.Z.).

AUTHOR CONTRIBUTIONS

J.O., A.-C.R., Y.M., Z.L., G.B., and N.Z. developed the research concept and optimized the protocol with critical input from Y.I. and M.S.; D.M.H. provided the immortalized astrocytes and HJ15.4 anti-apoE antibody; X.H. group performed the lipidomic analysis; J.O., Z.L., and N.Z. performed statistical data analysis; J.O., Z.L., and N.Z. created the figures; J.O. and N.Z. wrote the manuscript with critical input and revisions from all co-authors.

DECLARATION OF INTERESTS

D.M.H co-founded and is on the scientific advisory board of C2N Diagnostics. D.M.H consults for Genentech, Denali, Cajal Neurosciences, and Alector. G.B. is a current employee of SciNeuro Pharmaceuticals.

REFERENCES

1. Yamazaki, Y., Zhao, N., Caulfield, T.R., Liu, C.C., and Bu, G. (2019). Apolipoprotein E and Alzheimer disease: pathobiology and targeting strategies. Nat. Rev. Neurol. *15*, 501–518. https://doi.org/10.1038/s41582-019-0228-7.

2. Zhao, N., Liu, C.C., Qiao, W., and Bu, G. (2018). Apolipoprotein E, receptors, and modulation of Alzheimer's disease. Biol. Psychiatry *83*, 347–357. https://doi.org/10.1016/j.biopsych.2017.03.003.

3. Raulin, A.C., Martens, Y.A., and Bu, G. (2022). Lipoproteins in the central nervous system: from Biology to pathobiology. Annu. Rev. Biochem. *91*, 731–759. https://doi.org/10.1146/annurev-biochem-032620-104801.

4. Martens, Y.A., Zhao, N., Liu, C.C., Kanekiyo, T., Yang, A.J., Goate, A.M., Holtzman, D.M., and Bu, G. (2022). ApoE Cascade Hypothesis in the pathogenesis of Alzheimer's disease and related dementias. Neuron *110*, 1304–1317. https://doi.org/10.1016/j.neuron.2022.03.004.

5. DeMattos, R.B., Brendza, R.P., Heuser, J.E., Kierson, M., Cirrito, J.R., Fryer, J., Sullivan, P.M., Fagan, A.M., Han, X., and Holtzman, D.M. (2001). Purification and characterization of astrocyte-secreted apolipoprotein E and J-containing lipoproteins from wild-type and human apoE transgenic mice. Neurochem. Int. *39*, 415–425. https://doi.org/10.1016/s0197-0186(01)00049-3.

6. Morikawa, M., Fryer, J.D., Sullivan, P.M., Christopher, E.A., Wahrle, S.E., DeMattos, R.B., O'Dell, M.A., Fagan, A.M., Lashuel, H.A., Walz, T., et al. (2005). Production and characterization of astrocyte-derived human apolipoprotein E isoforms from immortalized astrocytes and their interactions with amyloid-beta. Neurobiol. Dis. *19*, 66–76. https://doi.org/10.1016/j.nbd.2004.11.005.

7. Liao, F., Zhang, T.J., Jiang, H., Lefton, K.B., Robinson, G.O., Vassar, R., Sullivan, P.M., and Holtzman, D.M. (2015). Murine versus human apolipoprotein E4: differential facilitation of and co-localization in cerebral amyloid angiopathy and amyloid plaques in APP transgenic mouse models. Acta Neuropathol. Commun. *3*, 70. https://doi.org/10.1186/s40478-015-0250-y.

8. Liao, F., Li, A., Xiong, M., Bien-Ly, N., Jiang, H., Zhang, Y., Finn, M.B., Hoyle, R., Keyser, J., Lefton, K.B., et al. (2018). Targeting of nonlipidated, aggregated apoE with antibodies inhibits amyloid accumulation. J. Clin. Invest. *128*, 2144–2155. https://doi.org/10.1172/JCI96429.

9. Tcw, J., Wang, M., Pimenova, A.A., Bowles, K.R., Hartley, B.J., Lacin, E., Machlovi, S.I., Abdelaal, R., Karch, C.M., Phatnani, H., et al. (2017). An efficient Platform for astrocyte Differentiation from human induced Pluripotent Stem cells. Stem Cell Rep. *9*, 600–614. https://doi.org/10.1016/j.stemcr.2017.06.018.

10. Wang, J., Wang, C., and Han, X. (2019). Tutorial on lipidomics. Anal. Chim. Acta *1061*, 28–41. https://doi.org/10.1016/j.aca.2019.01.043.

11. Zhao, J., Lu, W., Ren, Y., Fu, Y., Martens, Y.A., Shue, F., Davis, M.D., Wang, X., Chen, K., Li, F., et al. (2021). Apolipoprotein E regulates lipid metabolism and alpha-synuclein pathology in human iPSC-derived cerebral organoids. Acta Neuropathol. *142*, 807–825. https://doi.org/10.1007/s00401-021-02361-9.