SOP from the University of Maryland Brain and Tissue Bank

A Brain and Tissue Repository of the NIH NeuroBioBank

Submitted July 12, 2017

APOE Genotyping:

Restriction Fragment Length Polymorphism

I. DNA Extraction

- **Qiagen: DNeasy Blood & Tissue Kit**
  - **Collect 25 mg of cerebellum tissue**
  - **Homogenize tissue w/ ceramic beads & Bullet Blender**
  - **Extract DNA w/ Blood & Tissue Kit**
  - **Elute 150ul volume**
  - **Normalize a 20ul aliquot to 50 ng/ul (for PCR)**

II. PCR

- **Roche Expand High Fidelity PCR System**
  - **Template DNA (250 ng)**
  - **APOE primers (0.5uM)**
  - **10x buffer w/ $MgCl_2$---add additional 2mM MgCl2**
  - **DMSO [10%]**
  - **50 ul rxn**
- **PCR product 218 bp, verify on 2% Agarose gel (TBE)**

III. Enzyme Digestion

- **PCR precipitation & resuspension in PCR grade water**
- **CFO-I Digestion**

IV. RFLP- Anaylsis

- **10% Acrylamide TBE gel**
- **Analysis of APOE Alleles**

Genomic DNA Purification: DNeasy Blood & Tissue Kit

(modified extraction w/ Bullet Blender Homogenizer)

1. **Collect <25 mg of tissue in pre-cooled 1.5 ml tubes, store immediately at -80°C until ready to process.**
2. **Bring tissue to room temp.**
3. **Add 180 ul of ALT buffer.**
4. **Add ½ scoop (approx 6) 2.00 mm ceramic beads to tube.**
5. **Run the Bullet Blender homogenizer @ speed 3 for 1 min.**
6. **Transfer supernatant to new 1.5 ml tube---adding Protienase K, 20ul/tube.**
7. **Vortex, incubate 1 hour @ 56°C---vortex during incubation.**
8. **Let cool to room temp. Add 4ul/tube RNase A (100mg/ml), vortex to mix, incubate 2 min at RT.**
9. **Make a master mix of <u>buffer AL & 100% ethanol</u>: 200ul of each per sample. (for 18 samples make a 20x master mix—4ml of Buffer AL + 4ml of 100% ethanol---vortex master mix immediately).**
10. **After step #8, vortex each tube for 15 sec. Add 400ul/tube of master mix. Vortex.**
11. **Pipet mixture into DNeasy mini spin column placed in a 2ml collection tube (numbering spin tubes 1-18).**
12. **Centrifuge for 1 min @ > 6000 x g (8000 rpm). Discard flow-thru and collection tube.**
13. **Place spin column in new collection tube. Add 500 ul Buffer AW1. Centrifuge 1min @ 8,000 rpm. Discard flow-thru and collection tube.**

Genomic DNA Purification: DNeasy Blood & Tissue Kit

14. **Place spin column in a new 2ml collection tube, add 500 ul Buffer AW2, and centrifuge for 3 min @ 20,000 x g (14,000 rpm). Discard flow-thru and collection tube.** (If ETOH seen in column, spin again for 1min @ 14,000 rpm).
15. **Transfer spin column to a new 1.5 ml tube.**
16. **Elute the DNA by adding 50 [75 ul] ** of Buffer AE to center of spin column membrane. Incubate for 1 min. @ room temp. Centrifuge for 1min @ > 6,000 x g.**
17. **Repeat step 16 using same tube for second elution for a total volume of 100 [150 ul] ****
18. **Quantitate DNA on Nanodrop: measuring ng/ul; checking 260/280 ratio; and 260/230 ratios.**

<u>Electrophoresis:</u> (optional, check for DNA degradation)

19. **Run 0.5 ug of DNA in a 20ul volume on 0.5% agarose gel w/ 5ul of EtBr.**
20. **Run gel w/DNA ladder Lambda/Hind 3** (follow instructions, 1ul of MW + 1ul dye + 4ul of dd H_2O-- heating marker @65 ^{o}C for 5 min. Place on ice 3 min before running). Top band is 23KB.
21. **Run gel in 1 x TAE buffer for 1hour @ 70 volts. Expose gel for 2 sec, zoom picture with 3 bars.**

***Notes:**
Dneasy Blood & Tissue Kit (Qiagen # 69504) **Rnase A (100mg/ml)--(Qiagen # 19101)**
2.0 mm Zirconium Oxide Beads (Next Advance-# ZrOB20) **Bullet Blender Homogenizer (Next Advance)**
Lambda DNA/Hind III marker (Fermentas # SM0101)

**** Elute with 75 ul X 2= 150 ul total volume for cerebellum tissues**

**** 11/2/12---We changed collection source from cerebral cortex to cerebellum. We found a 5-10 fold increase in DNA concentration.**

II. PCR

Materials:

- **Roche Expand High Fidelity PCR System (50 ulrxn)**
- **10mM dNTP mix**
- **APOE primer set [10 pmole/ul= 10 uM]**
- **Template DNA [250 ng]. Samples normalized to 50 ng/ul, use 5 ul of template. Adjust volume to template concen.**
- **DMSO**
- **PCR grade water**

PCR conditions: Initial denaturing step: 95°C for 5 min. Then program 35 cycles: 95°C for 1 min, 65°C for 1 min, 72°C for 1min. Final extention step 72°C for 4 min, then 4°C ∞. Expect 218 bp PCR product.

PCR program #8 APOE in Techne 3000 Thermocycler. NOTE: prepare negative control w/o DNA template

Prepare PCR in 2 steps: (all reagents on ICE)

Master Mix #1

1x		10x	20x	Final Conc.	Control
1.0 ul	dNTP	10.0 ul	20.0 ul	[2mM each]	"
2.5ul	Primer up	25.0ul	50.0 ul	[0.5 uM]	"
2.5ul	Primer dwn	25.0ul	50.0 ul	[0.5 uM]	"
14.0ul ul/tube	PCR water (add 1st)	140.0 ul	280.0 ul	(pipette u/d shake dwn) ->-> Aliquot 20	
5.0ul	template DNA*	5.0ul (50 ng/ul)	[250 ng] adjust vol to ng/ul		{add 5ul of water}

25.0 ul (Ttl vol) *(add DNA after aliquot 20ul/tube of master mix #1)

Master Mix #2

1x		10x	20X	Final Conc.	
10.25 ul	PCR water	102.5 ul	205 ul	-----	
5.0 ul	10x buffer w/Mg	50.0 ul	100 ul	[1x]	
5.0 ul	DMSO	50.0 ul	100 ul	[10%]	
4.0 ul	MgCl	40.0 ul	80 ul	[2 mM]	
0.75 ul	Enzyme* [3.5U/ul]	7.5 ul	15 ul	[2.6 U/rxn]	*Add last---keep on ICE
25.0 ul		250.0 ul→→→→(mix well--aliquot 25ul/tube)			

Run 15ul PCR product + 3ul of 6x loading dye on 2% agarose gel in 1x TBE buffer with N.E.B. 100bp ladder. Run @ 100 volts for 30-45 min. Expect PCR product of 218 bp size. Expect no band for negative control. 2% gel [1.2 g/60 ml, w/ 5ul Etbr].

III. Restriction Enzyme Digestion

A. PCR Precipitation

35ul of PCR product (transfer remaining PCR to 0.5 ml tube)

3.5 ul of 3M Sodium Acetate, pH 5.2 (1/10 vol)

100 ul of 100% ethanol (2.5vol)

Vortex, incubate -20^{o}C for at least 2hours or -80^{o}C for 30 min.

Centrifuge 14,000 rpm for 10 min.

Carefully aspirate the supernatant using a narrow pipet (gel loading pipet tip). You will not always see the pellet so be aware of the tube orientation & which side the DNA is likely to be found. Leave tube open to let air dry for about 15 min. Resuspend DNA in 35 ul PCR grade water (if PCR band easily seen resuspend in 35 ul, adjust as needed).

B. CFO-I Enzyme digestion: Master Mix: Digest 13 ul of PCR product.

	1x	Reagent	10x	20x	
	1.5ul	10x buffer	15.0 ul	30.0 ul	
	0.2 ul	BSA [10ug/ul]	2.0 ul	4.0 ul	
	0.5ul	CFO-I enzyme	5.0 ul	10.0 ul	
	2.2ul volume->->->		*aliquot 2.2 ul/ tube		*add to tube after PCR product
+	13.0ul PCR product		add 13.0 ul of PCR product		(store remaining PCR @-20^{O}C)

15.2 ul total volume

Incubate 37^{o}C for 90 min. Add 5ul of DNA loading dye (Biorad 5x)— add directly to restriction digest. Mix well, load 14ul on 10% acrylamide TBE gel. Run in 1x TBE @ 100 volts, run until dye front is at bottom of gel, about 75 min. Note: remove green tape @ bottom of acrylamide gel, rinse wells after removing comb, before loading gel.

Load gel: (OR)

Lane # 1:	20 bp DNA ladder (Bio-rad)	Lane 1	20 bp DNA ladder
Lane # 2:	uncut PCR product (220 bp)	Lane 2:	1 known allele (#4924-E2/E3)
Lane # 3-8:	six unknown control samples	Lane 3-10:	8 unknown controls
Lane # 9-10:	2 known APOE alleles		

Stain gel with Ethidium bromide (EthBr) 10 ul/ 200ml dd water for 15 min, destain 2x for 5min. with fresh dd water. Lay gel flat on UV light box. Develop gel using autoexposure & noise reduction.

IV. RFLP-APOE Allele Anaylsis

According to restriction map of APOE amplified product, the CFO-1 cuts produce following band sizes:

E4: Cuts at codon 112 and 158; fragments sizes: 72

48

25

20

E3: No cut at codon 112, cuts at 158, fragment sizes: 91

48

25

20

E2: No cut at codon 112 or 158; fragment sizes: 91

73

55

48

20

You can have several combinations: E4/E4

E3/E4

E2/E3

E3/E3

Bp	E4/E4	E3/E3	E3/E4	E2/E3	E2/E2	E2/E4
91		—	—	—	—	—
73				—	—	—
72	—		—			—
55*				—	—	—
48	—	—	—	—	—	—
25	—	—	—	—		—
20	—	—	—	—	—	—

RESTRICTION MAP OF APOE AMPLIFIED PRODUCT:

underlined sequence is the primer, the red sequences are where the CfoI cuts.

TCCAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGAC

APOE-UP

GTGCGCGGCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCC

112

AGAGCACCGAGGAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCG

TAAGCGGCTCCTCCGCGATGCCGATGACCTGCAGAAGCGCCTGGCAGTGTAC

158 **APOE-DOWN**

CAGGCCGGGGC

1 20 36 55 127 145 193 218

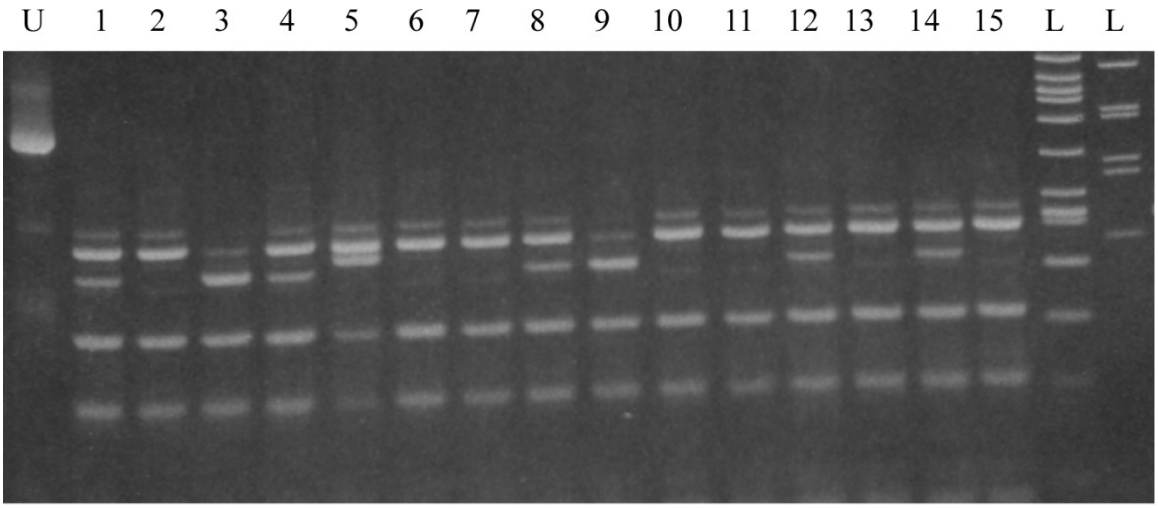
E4: Cuts at codon 112 and 158; fragment sizes- **72**, **48**, **25**, **20**

E3: No cut at codon 112, cuts at 158; fragment sizes- **91**, **48**, **25**, **20**

E2: No cut at codon 112 or 158; fragment sizes- **91**, **73**, **55**, **48**, **20**

ApoE Genotyping from Buccal Swabs

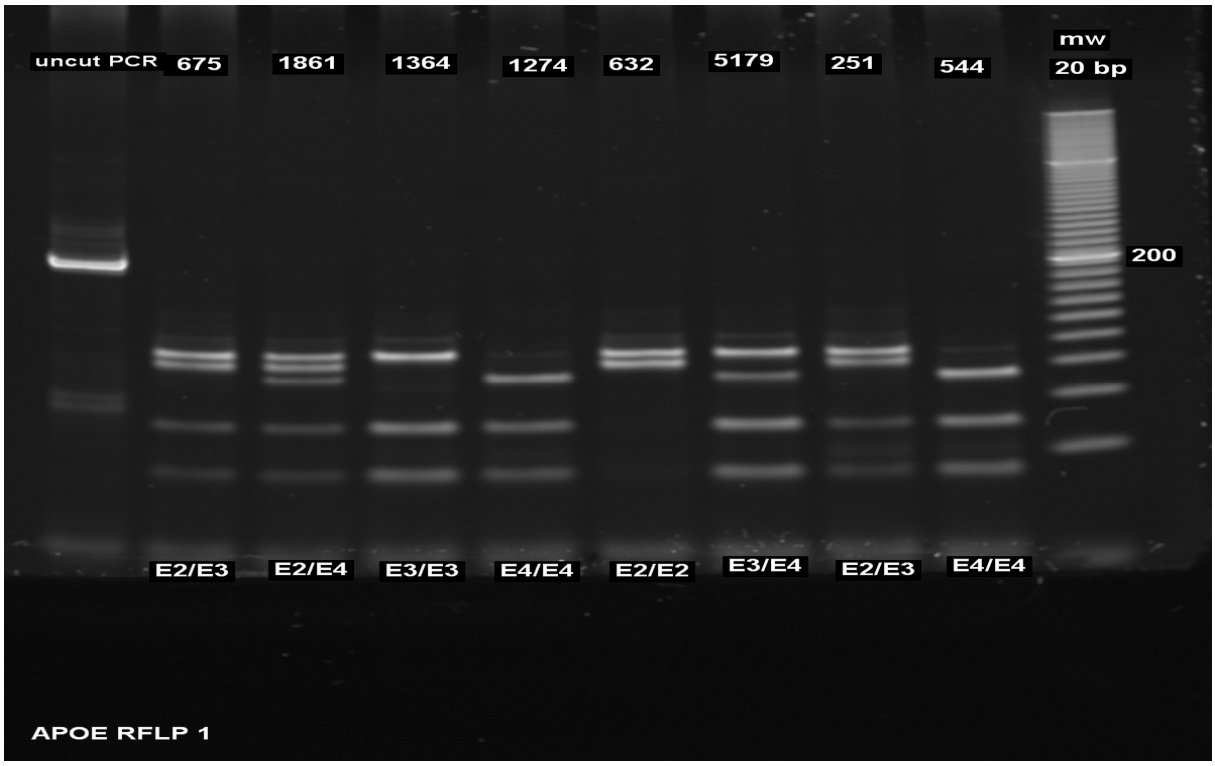
DNA is extracted using the EpicentreQuickExtract DNA extraction protocol. Briefly, the buccal swab is swirled in 300µl QuickExtract DNA Extraction Solution, vortexed and incubated at 65°C for 30 minutes, followed by an incubation at 98°C for 16 minutes, vortexing twice during the incubation. A portion of the ApoE gene was amplified using the Roche Expand High Fidelity PCR system. Following PCR the amplified product was analyzed by restriction with CfoI (Promega) and electrophoresis in an 8% acrylamide gel in Tris/Borate/ EDTA buffer. An example is shown below:

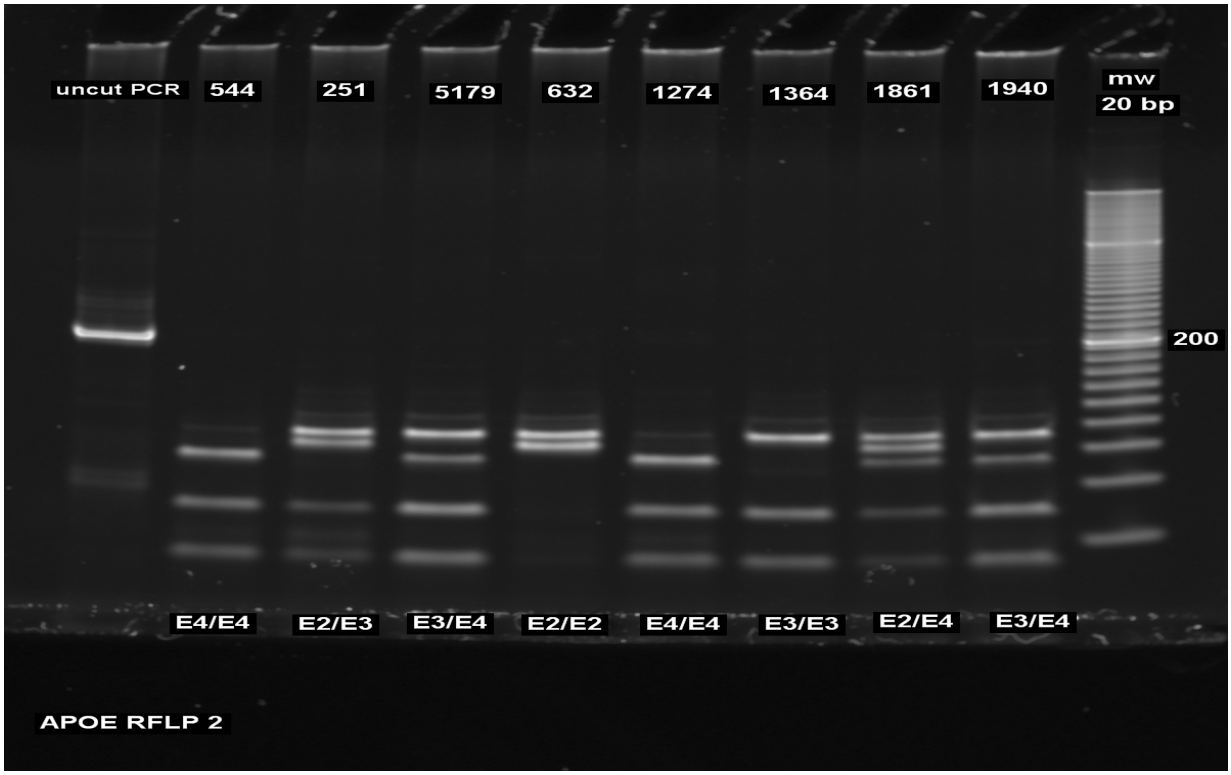


Sample	Genotype
1,4,8,12,14:	3/4
2,6,7,10,11,13,15	3/3
3,9	4/4
5	2/3

U: uncut
L: molecular weight ladder

Retrieved from Sandy Richardson @ Case Western


uncut PCR
675
1861
1364
1274
632
5179
251
544
mw
20 bp
200
E2/E3
E2/E4
E3/E3
E4/E4
E2/E2
E3/E4
E2/E3
E4/E4
APOE RFLP 1


uncut PCR
544
251
5179
632
1274
1364
1861
1940
mw
20 bp
200
E4/E4
E2/E3
E3/E4
E2/E2
E4/E4
E3/E3
E2/E4
E3/E4
APOE RFLP 2