Sanger sequencing for genes causing intellectual disability

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ABSTRACT

Sanger sequencing was performed on available samples from all affected family members to determine whether the potential variant in the known genes of autosomal recessive intellectual disability co-segregated with the disease phenotype. Initially we performed a touchdown PCR. The amplified products were then purified using Sephadex Clean up and were consequently sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, USA). The resulting sequencing reactions were then purified Sephadex Clean up method. Capillary sequencing was performed in a Genetic Analyzer 3130 (Applied Biosystems, Thermo Fisher Scientific, USA) and the data were analyzed using Sequencing Analysis software Sequencer 5.0.

EXTERNAL LINK

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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PROTOCOL STATUS

Working

GUIDELINES

1. Keep all reagents protected from light until you are ready to use them.
2. Minimize freeze-thaw cycles.

MATERIALS

NAME	CATALOG #	VENDOR
dNTPs		
Water refers to sterilized deionized water		
MgCl2		
Water		
Primer		
forward primer (25 uM)		
reverse primer (25 uM)		
illustra Sephadex G-50 DNA Grade	View	Ge Healthcare
Taq DNA Polymerase PCR Buffer (10X)	View	Invitrogen - Thermo Fisher
Platinum™ Taq DNA Polymerase	View	Invitrogen - Thermo Fisher

NAME	CATALOG #	VENDOR
MultiScreen Column Loader	View	Millipore Sigma
AcroPrep™ Advance 96-Well Filter Plates for Aqueous Filtration	View	Vwr
MicroAmp™ Clear Adhesive Film	View	Applied Biosystems
BigDye™ Terminator v3.1 Cycle Sequencing Kit	View	Applied Biosystems
BigDye™ Terminator v1.0 & v3.0 5X Sequencing Buffer	View	Applied Biosystems
QuickStep™2 SOPE Resin	View	Edge Bio

Normalize the DNA samples.

1 The DNA concentration per sample is 20 ng/µL

PCR

2 Calculate the number of reactions to be performed for each assay, including recommended controls.

Component	Working Concentration	Final Concentration	25 ul reaction
10x Buffer	10x	1x	2.5 ul
dNTPs	2 mM (each)	200 uM(each)	2.5 ul
MgCl2	50 mM	1.5 uM	0.75 ul
Taq	5 units/ul	1 unit/ul	0.2 ul
Forward Primer	20 uM	0.48 uM	0.7 ul
Reverse Primer	20 uM	0.48 uM	0.7 ul
DNA	n/a	20 ng/ul	1-2 ul
H2O	n/a	n/a	To 25 ul

NOTE

Use negative and positive control. Prepare excess volume to account for pipetting errors.

Place the plate/tubes in a PCR instrument. Use the thermal cycling conditions specified.

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Hot Start	94°C	3 minutes	1 cycle
Denaturation	94°C	5 sec	\
Annealing	65°C	30 sec	---2 cycles
Extension	72°C	30 sec	/
Denaturation	94°C	5 sec	\
Annealing	63°C	30 sec	---2 cycles
Extension	72°C	30 sec	/
Denaturation	94°C	5 sec	\
Annealing	61°C	30 sec	---2 cycles
Extension	72°C	30 sec	/
Denaturation	94°C	5 sec	\
Annealing	59°C	30 sec	---2 cycles
Extension	72°C	30 sec	/
Denaturation	94°C	5 sec	\
Annealing	57°C	30 sec	---2 cycles
Extension	72°C	30 sec	/

Denaturation	94°C	5 sec	\
Annealing	55°C	30 sec	---32 cycles
Extension	72°C	45 sec	/
Final Extension Hold	72°C	7 min	1 cycle
	4°C	∞	

Make two Sephadex Plates (number of wells adjusted for a number of samples to be sequenced)

4 1. Add dry Sephadex G-50 (VWR cat. 95016-896) to Millipore (cat. #MACL09645) 45 ul column loader (black mold plate)
2. Place a Pall Corportation multi-well plate (VWR cat. 97052-098) on top of the black mold plate
3. Carefully turn both plates over and tap the mold plate causing Sephadex to fall into wells
4. Add 300 ul of dH2O to each well
5. Cover plate with adhesive seal (Applied Biosystems cat. 4306311). Write date on plate
6. Allow plates to hydrate for 2 hours before use. Plates can stand in room temperature for 2 days. Can be stored at 4°C for two weeks (if stored at 4°C the plate must incubate at room temperature for 2 hours before use)

PCR Purification (SOPE resin [Edge Biosystems cat. 72418] , combined with Sephadex Plate)

5 1. Remove plate from PCR machine
2. Sephadex must be brought to room temperature, and adhesive seal removed
3. Place Sephadex plate on top of 96-well
4. Centrifuge sephadex plate for 5 min at 850 x g and dispose of the eluate
5. Bring PCR products to volume of 20 ul (should be what is remaining in PCR tube following Gel Run)
6. Add 4ul of SOPE resin directly to the PCR product mix thoroughlyby tip mix
7. Pipette the entire SOPE/PCR reaction mixture into the center of the wells of the Sephadex plate making sure the fluid runs into column
8. Stack the loaded Sephadex plate on a 96-well plate (Life Technologies cat. 4346907)
9. Tape the plates together
10. Centrifuge for 5 minutes at 850 x g and Retain Eluate, dispose of sephadex plate
11. If product will not be used immediately it can be stored at 4°C overnight or -20°C long term

BigDye Sequencing Reaction (optimized for Applied Biosystems 3130)

6 Calculate the number of reactions to be performed for each assay

Component	Volume Added
BigDye Terminator Ready Reaction Mix	0.75 ul
5x Sequencing Buffer	2 ul
Template (10-30ng)	0.75 ul
Primer (one direction) at 0.1ug/ul)	0.75 ul
Deionized Water	5.75 ul
Total Volume (per well)	10 ul

BigDye Sequencing Program

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96°C	1 min	
96°C	10 sec	
50°C	5 sec	25 cycles
60°C	4 min	
4°C	Hold	

Dye Terminator Removal (Sephadex Plate)

8 1. Remove plate from Thermal Cycler and cover with aluminum foil
2. Bring Sephadex plate to room temperature remove adhesive seal
3. Place Sephadex plate on top of 96-well “waste” plate
4. Centrifuge for 5 minutes at 850 x g and dispose of the eluate

5. Wash the columns
   a. Add 150 ul dH20 to each well
   b. Centrifuge for 5 minutes at 850 x g and dispose of eluate
6. Pipette 10 ul of dH2O directly in sequencing reactions
7. Transfer entire sequencing reaction volume (approximately 20 ul) directly to sephadex columns (load to center of the sephadex column without touching the column.
8. Stack the Sephadex plate on top of a 96-well ABI sequencing plate (cat4346907)
9. Tape the plates together
10. Centrifuge for 5 minutes at 850 x g and Keep Eluate
11. Pipette 20 ul of dH20 directly into any empty wells that will be included in run
    a. 3130 sequencer has 16 capillaries per run (there must be liquid in each well)
12. Seal the Plate with Adhesive tape, cover with aluminum foil, and label appropriately
13. Eluate can be stored at 4°C overnight, and -20°C for two days
14. For optimal results sequence immediately or asap