

Microsatellite Library Protocol

Last Update 11/26/03 D Drown
Modified from T. Garner Protocol

Outline of Protocol

DNA Extraction (1 overnight [optional], 3 hrs setup)

Digestion and Size Fractionation (8 hours)

Linker Creation (15 minutes)

Linker Ligation (15 minute setup, 1 overnight)

DNA Extraction (total time 3 hrs (plus overnight for PK))

DNAzol Genomic Isolation Reagent (Molecular Research Center, Inc Cat No. Dn 127)

1. Obtain tissue (25-50 mg). If concerned about shearing DNA digest tissue with standard PK digestion. (PK Digest To tissue add 500 uL DNAzol and 5 uL (10mg/mL) Proteinase-K. Incubate at RT overnight)
2. Add 1 mL DNAzol (500 uL if PK digest) and gently homogenize tissue.
3. Incubate at RT for 10 min
4. Centrifuge for 10 min at 10,000g to sediment the homogenate. If you extracted muscle tissue there will be a considerable amount of polysaccharides (snot).
5. Remove supernatant to a fresh tube.
6. Add 500 uL of 4 C 100% EtOH and mix by inversion
7. Incubate at 4 C for 30-60 min to precipitate DNA.
8. Pellet DNA at 5000 g for 5 min (longer may be better?)
9. Remove supernatant and wash twice with 825 uL of 75% EtOH spinning at 1000g for 2 min between each wash.
10. After final wash, dry pellet by inverting tube and waiting (aprox 1 hour)
11. Resuspend in 100 uL H₂O
12. Spec DNA to obtain concentration (spec X 50 X total volume mL)/sample in uL = ug/uL

Digestion and Size Fractionation

1. Digest extracted genomic DNA with TSP 509 I restriction enzyme (New England Biolabs #R0576S 1000 U). Add to a 1.5 mL eppendorf tube:
 - 35-50 μg DNA
 - 5 μl (50 units) TSP 509 I restriction enzyme
 - 10 μl of 10x buffer
 - enough water to total 100 μl .
2. Incubate at 65°C for 4-6 hours. Stop reaction with 5 μl of 1X TAE Buffer and cool on ice.
3. Pour an 0.8% agarose gel using 1X TAE and place in refrigerator to solidify. Use largest, fattest combs available to form wells.
4. Load digest into 1-2 lanes of agarose gel and run at 80 V for 30 minutes. **Note clean chamber and use fresh TBE buffer to avoid contamination**
5. Stain gel with ethidium bromide.
6. Visualize gel and take picture for your records.
7. Slice out bands corresponding to 500-1000 bp. Be sure to use sterile scapel and trim away as much agarose as possible. Gel slice must be under 100 μL
8. Purify gel slices using Montage Gel Extraction Kit (Millipore, Cat No. LSKGEL050) by putting in gel nebulizer.
9. Centrifuge at 5000 g for 10 minutes.
10. Discard the nebulizer and the sample filter.
11. Spec DNA

Linker Creation (Can be done ahead of time)

1. Order the following oligos:
TSPADSHORT: 5' CGG AAT TCT GGA CTC AGT GCC 3'
TSPADLONG: 5' AAT TGG CAC TGA GTC CAG AAT TCC G 3'
2. Dilute to 50 uM stocks of each oligo
3. In a 200 uL PCR tube add:
10 uL 50uM TSPADSHORT
10 uL 50 uM TSPADLONG
0.8 uL 5M NaCL
79.2 uL TE
4. Incubate on a thermocycler with the following protocol:
95°C for 3 min
65°C for 2 min
45°C for 2 min
25°C for 1 min
4°C hold

Linker Ligation

1. In a 200 uL PCR tube add:
 - 2-3 ug of size fraction DNA
 - 10 uL of Linker
 - 10 uL 10X ligase buffer
 - 36 U T4 DNA ligase (Fermentas Cat. No. EL0015)
 - add ddH₂O until final volume is 100 μ l
2. Ligate overnight on PCR machine set at 16°C.
3. Clean ligate with Millipore Ultrafree Centrifugal Filters (Millipore Cat. No. UFC810024). Add entire ligate reaction to a single tube and filter unit. Add 3 mL of ddH₂O.
4. Centrifuge at 3000 g for 2 min.
5. Check tube if more than 100 μ l of ligate remains in filter repeat until centrifugation until only 100 μ l remains in filter. Pipette remaining 100 μ l into a new tube.

Size fraction-linker ligation test PCR

1. Set up PCR reactions (1 with linker ligation and a negative control). To each rxn:
 - 14.25 H₂O
 - 2.5 μ l 10x Buffer
 - 2.5 μ l dNTPs
 - 2.5 μ l MgCl₂
 - 2.5 μ l TSPADSHORT (10 μ M)
 - 0.25 Taq
 - 1 μ l template DNA
2. Run on PCR machine with following program
 - 72°C 5 min
 - 30 cycles of
 - 94°C 1 min
 - 55°C 1 min
 - 72°C 1 min
 - 24°C 2 min
3. Run amplified product out on a 1% agarose gel at 90V for 45 minutes. Visualize as usual. Product should be b/w 500-1000 bp in size (may appear as a large smear).

Biotinilation of microsatellite probe

1. Order repeat oligo (aprox 30 bp) and dilute to 40 uM
2. Add to a 1.5 mL tube:
 - 5 μ l probe primer (40 μ M)
 - 8 μ l COCL2 (TT kit)
 - 2 μ l d-UPT Biotin (Roche Applied Science Cat. No. 1093070)
 - 8 μ l 5x Terminal transferase Buffer (TT Kit)
 - 0.5 μ l Terminal Transferase (Roche Applied Science Cat. No. 3333566)
 - 16.5 μ l ddH2O
3. Incubate at 37°C water bath for 25 min.
4. Remove from water bath, add 4 μ l of 3M sodium acetate, 100 μ l of 100% ethanol, and freeze overnight at -20°C
5. Centrifuge at 13,000 g for 30 minutes.
6. Remove ethanol, wash the pellet (impossible to see) with 70% ethanol
7. Centrifuge at 13,000 g for 5 min
8. Invert tube to dry
9. Suspend in 100 μ l H2O. Store at 4 C until attached to Dynabeads

Prepare Dynabeads

1. Pipette 200 μ l of beads into 1.5 mL tube. Place tube in magnet concentrator and pipette off storage solution.
2. Add 200 μ l of PBS/BSA solution to beads. Finger vortex.
3. Place back into magnet concentrator. Pipette off PBS/BSA.
4. Repeat wash 2 more times (total of 3 washes).
5. Add 200 μ l of B&W solution. Finger vortex.
6. Divide beads into two 100 uL aliquots and store at 4 C
7. To attach probe, incubate at beads RT for 1 hour. Add entire probe to solution and incubate at RT for 1 more hour. Store at 4 C

PCR enrichment of size fraction-linker ligation

1. Set up 40 PCR reactions using following recipe”
 - 14.25 H₂O
 - 2.5 μ l 10x Buffer
 - 2.5 μ l dNTPs
 - 2.5 μ l MgCl₂
 - 2.5 μ l TSPADSHORT (10 μ M)
 - 0.25 Taq
 - 1 μ l template DNA
2. Run on PCR machine with following program:
 - 72°C 5 min
 - 12 cycles of
 - 94°C 1 min
 - 55°C 1 min
 - 72°C 1 min
 - 24°C 2 min
3. When PCR reactions are finished combine all 40 rxns in a Millipore Ultrafree tube and add approximately 3 mL of ddH₂O to tube
4. Centrifuge at 3000 g for 2 min. Collect the 50-100 uL into a new tube

Hybridize probe to microsatellites

1. Make up the following solutions:
 - 1 15 mL tube of 5x SSC, 0.1% SDS
 - 1 15 mL tube of 10X SSC, 0.2% SDS
 - 2 15 mL tube of 2X SSC, 0.1% SDS
 - 1 15 mL tube tube with 900 μ L 1 M TE and 10 μ l 5 M NaCl
 - 2
2. Preheat hybridization oven to 65°C.
3. Put the 10X SSC, 0.2% and 1 2X SSC, 0.1% tubes in oven to preheat.
4. Disassociate Enriched PCR by heating at 95 C for 5 min then snap cool on ice
5. Wash Bead-Probe: Concentrate with magnet remove liquid, add 200 uL B&W solution and finger Vortex
6. Repeat above wash
7. Wash once more with 200 uL RT 5X SSC, 0.1%SDS
8. Remove liquid with magnet and add 150 uL of warm 10X SSC, 0.2% SDS and PCR products.
9. Tape tube to rotor in oven and incubate at 65 C for 5 hours
10. Remove from incubator (do not turn off)
11. Remove liquid using magnet concentrator
12. Add 200 uL of RT 2X SSC, 0.1%SDS
13. Rotate by hand for 5 minutes
14. Repeat wash and 5 minute mixing
15. Remove liquid using magnet concentrator
16. Add 200 uL of Warm 2X SSC, 0.1% SDS
17. Rotate in incubator for 10 minutes at 65 C
18. Remove liquid with magnet concentrator
19. Add 200 uL of TE/NaCl solution
20. Remove liquid using magnet concentrator
21. Add 200 uL of TE