

DeWoody's Microsatellite Cloning Protocol (Spring 2002)

Based in part on the SNX-linker protocol by Hamilton et al. 1999 (*Biotechniques* 27:500) and on Travis Glenn's "Dynabeads2002" protocol

Most (hopefully, all) requisite reagents are represented in bold, red text at first mention

Step 1—DNA extraction (see Sambrook et al. 2001 *Molecular Cloning* Chap. 6, protocol 5)

1. Collect eraser-sized tissue sample from a heterogametic individual
2. Mince with a razor blade and transfer to a 15-ml conical tube
3. Add 2000 μ l **SNET** (20 mM Tris-Cl (pH 8.0); 5 mM EDTA (pH 8.0); 400 mM NaCl; 1% (w/v) SDS)
4. Add **Proteinase K** to a final concentration of 400 μ g/ml (i.e., use 80 μ l of 10mg/ml soln)
5. Digest to completion (2-18 hrs) at 55°C with gentle mixing in parafilm-sealed tube
6. Equitably split digestion into four microfuge tubes
7. Add equal volume TE-equilibrated **phenol** to each and incubate/mix by inversion for 10'
8. Spin at high speed for 5 minutes to separate phases
9. Carefully collect aqueous (top) phase using pipettor
10. Add equal volume of **PCI** (25:24:1) and incubate/mix by gentle inversion for 10'
11. Spin at high speed for 5 minutes
12. Collect aqueous (top) phase
13. Add equal volume of **CI** (24:1) and incubate/mix by gentle inversion for 10'
14. Spin at high speed for 5 minutes
15. Collect aqueous (top) phase
16. Repeat 13-15
17. Add equal volume of 100% **isopropanol** (High quality DNA will often spool out at this step)
18. Freeze at -80°C for 15-20 minutes
19. Spin at high speed for 15' at 4°C
19. Carefully decant supernatant, rinse DNA pellet with 0.2 ml **70% ethanol**
20. If DNA doesn't spool out of solution, spin 15' at 4°C
21. Air dry the DNA (at room temp) for 15-20'
22. Resuspend each pellet in ~50 μ l T.E (10 mM Tris-Cl; 0.1 mM EDTA pH 8.0) and store at 4°C
23. Run 2 μ l DNA in 1% agarose gel to check concentration

Step 2—DNA processing

1. Digest (at 37°C) 1 μ g genomic DNA with suites of restriction enzymes to get *appropriate sized fragments* (i.e., between ~400-1200 bp). [Never let enzyme volume exceed 1/10 digestion volume or the glycerol in the enzyme storage buffer will inhibit enzyme activity. Tap spin enzymes immediately upon removal from the freezer.]

Rsa I and Nhe I

- 5 μ l DNA
- 3 μ l 10X NEB buffer #2
- 1.0 μ l *Rsa I* (10 Units)
- 1.0 μ l *Nhe I* (5 U)
- 20.0 μ l sterile water

Hae III, Rsa I, and Nhe I

- 5 µl DNA
- 3 µl 10X NEB buffer #2
- 0.5 µl *Hae III* (25U)
- 1.0 µl *Rsa I* (10 U)
- 1.0 µl *Nhe I* (5 U)
- 19.5 µl sterile water

Rsa I, Alu I, and Nhe I

- 5 µl DNA
- 3 µl 10X NEB buffer #2
- 1.0 µl *Nhe I* (5 U)
- 1.0 µl *Rsa I* (10 U)
- 1.0 µl *Alu I* (10 U)
- 19 µl sterile water

2. Run on 1% agarose gel to check completeness of digestion; run undigested DNA as control
3. Pick enzyme combination that gives ~500 bp fragments and digest ~10 µg at 37°C.

- 50 µl DNA (at 200 ng/µl, or roughly 10 µg)
- 10 µl 10X NEB buffer #2
- enzyme (>10 units)
- enzyme (>10 units)
- enzyme (>10 units)
- q.s. with sterile water

Note: In Jan. 2002, three different libraries (otter/salamander/kangaroo rat) constructed in the DeWoody lab all worked best with *Hae III*, *Nhe I*, and *Rsa I*.

4. Inactivate enzymes by incubating for 20 minutes at 65°C, then run a portion of the digest on a 1% agarose gel to test digestion (run undigested genomic DNA as a control).
5. Digest with **mung bean nuclease** for 30' at 30°C to blunt the *Nhe I* sticky ends
 - add 1.0 µl NEB mung bean nuclease (10U) to entire restriction digest
6. Purify genomic DNA using **Qiaquick column** and elute in 40 µl elution buffer EB
7. Dephosphorylate 5' ends of digested DNA by adding calf intestinal phosphatase (**CIP or CIAP**)
 - 40 µl eluted DNA
 - 5 µl 10X alkaline phosphatase buffer (Takara Biomedical #2250A)
 - 2 µl CIP (40U)
 - 3 µl sterile water
 - incubate at 37°C for 15' then 50°C for 15'
8. Purify genomic DNA using Qiaquick column and elute in 30 µl elution buffer EB

Step 3—Ligation

1. Set up ligation of restriction fragments to linkers
 - 11.7 µl double-stranded **SNX linkers** (5 µM each; see Hamilton et al. 1999)
 - 10.0 µl digested & dephosphorylated DNA (from #8 above)
 - 3.0 µl NEB buffer #2
 - 0.3 µl 100X BSA
 - 3.0 µl **rATP** (10 mM stock; thus, 1 mM final concentration)
 - 1.0 µl **T4 DNA ligase** (2000U)
 - 1.0 µl **Xmn I** (20U)

- incubate overnight for 30 cycles of 30' at 16°C/10' at 37°C
 - heat at 65°C for 20'
2. PCR-test of ligation
- 2.0 µl ligation template
 - 5.0 µl 10X Fisher *Taq* buffer B
 - 8.0 µl **dNTPs** (200 µM each)
 - 3.0 µl **MgCl₂** (1.5 mM final concentration)
 - 1.0 µl **Taq** (5U; Fisher)
 - 4.0 µl SNX double-stranded linker
 - 27.0 µl H₂O
 - Control reaction contains linker and digested/dephosphorylated DNA
 - 45/60/60 at 60°C for 35 cycles preceded by 5' at 95°C, followed by 2' at 72°C
 - Run 10 µl of PCR product in 1% gel with uncut genomic and size standard—a successful PCR will produce a smear the same size as the original digest.

Step 4—Enrichments

1. Hybridizations (one tetranucleotide enrichment, one dinucleotide enrichment; two 0.2 ml tubes)
- TETRA TUBE
 - 13.0 µl linker-ligated DNA
 - 3.0 µl of 10 µM (i.e., 30 pmol) of **biotinylated microsat oligo** (GATA)₇
 - 3.0 µl (GATC)₇
 - 3.0 µl (GACA)₇
 - 25 µl 2X hybridization solution (**12X SSC, 0.2% SDS**)
 - 3 µl H₂O
 - DI TUBE
 - 13.0 µl linker-ligated DNA
 - 3.0 µl of 10 µM biotinylated (GT)₁₂
 - 3.0 µl of 10 µM biotinylated (CT)₁₂
 - 25 µl 2X hybridization solution (12X SSC, 0.2% SDS)
 - 6.0 µl H₂O

Program thermal cycler with the following profile: First, denature each tube (n=2) of probe/DNA mixture for 10' at 95°C. Then go to 75°C for 1', then go to 70°C and step down 0.2°C every 10 seconds (5 step downs per degree x 20 degrees = 99 cycles) [i.e., 70°C for 10", 69.8°C for 10", 69.6°C for 10", down to 50.2°C], then stay at 50°C for 10'. Next, ramp down 0.5°C every 10 seconds for 20 cycles (i.e., 50°C for 10", 49.5°C for 10", 49.0°C for 10", ...down to 40.0°C). Finally, ramp down to 4°C for infinity. [The entire program should take about 75 minutes to complete—that is, to reach the 4°C step.]

2. Prepare **M-280 Streptavidin-coated Dynabeads** (Dynal, Inc)
- Mix beads and aliquot 300 µl (3000 µg of beads, or ~**600 µg per oligo**) into 1.5 ml tube
 - place tube into **Magnetic Particle Concentrator** (MPC) for 1 minute
 - while tube remains in MPC, remove and discard supernatant
 - add 300 µl binding & wash (**B&W**) **buffer [10 mM Tris-Cl, 1 mM EDTA, 1M NaCl]**
 - while tube remains in MPC, remove and discard supernatant
 - add 300 µl B&W buffer and mix out of MPC

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- aliquot 150 µl into 2 individual 1.5 ml “bead” tubes
- quick spin hybs (step #1) and add entire reactions plus 100 µl water to each bead tube
- Parafilm tubes, then incubate in rotating hybridization oven for ~2 hours at 33°C

3. Washes

- Set heat blocks at 40°C, 50°C and 95°C
- Place tubes in magnet, wait 1 minute, remove supernatant
- Wash for 2' at room temp with 300 µl **6X SSC, 0.1% SDS**, magnetize, remove super
- Repeat
- Wash for 2' at 40°C with 300 µl **1X SSC, 0.1% SDS**, magnetize, remove supernatant
- Repeat
- Wash for 2' at 50°C with 300 µl 1X SSC, 0.1% SDS, magnetize, remove supernatant
- Repeat
- Elute genomic DNA by adding 200 µl of “**TLE**” (10 mM Tris, 0.1 mM EDTA)
- Incubate at 95°C for 10', then magnetize and save supernatant (i.e., enrichments!)
- Add 60 µl TLE to beads, then clearly label (!) and store them at -20°C

4. Ethanol precipitate supernatants

- add 22 µl 3M NaOAc
- add 500 µl ice-cold 100% ethanol and mix well; freeze for >15'
- spin full speed for 10'
- carefully decant supernatants and slowly add 400 µl of 70% ethanol
- spin full speed for 2'
- decant supernatants and air dry until EtOH completely evaporates
- resuspend pellets in 40 µl TLE and store at 4°C

5. Amplify microsatellite-enriched DNA

- 10 µl enriched DNA
- 5 µl 10X *Taq* buffer
- 8.0 µl dNTPs
- 3.0 µl SNX-F
- 1.0 µl *Taq* DNA polymerase
- 3.0 µl MgCl₂
- 20 µl water
- 45/45/60 at 58°C for 32 cycles preceded by 3' at 95°C, followed by 10' at 72°C
- Run 10 µl of PCR on gel along with 100 bp ladder

6. If it worked, Qiagen clean-up PCR products; elute in 50 µl EB

At this point, the enrichment procedure can be repeated (i.e., go back to Step 4 #1). Simply use the purified PCR product instead of the ligation reaction. We did this with all three libraries (otter, salamander, kangaroo rat) made in January of 2002.

Step 5—Cloning

1. Digest eluants (tetras and di's) with *Nhe I* to prep ends for subsequent *Xba I* ligation
 - 1.0 μ l *Nhe I* (5U)
 - 43.5 μ l eluant
 - 0.5 μ l BSA (100X stock; 0.1 mg/ml final concentration)
 - 5 μ l NEB 10X Buffer #2
 - incubate at 37°C for >2 hr
 - Qiagen clean-up and elute in 30 μ l EB buffer
2. Digest 3 μ g **pBS II SK(+)** **Bluescript** with *Xba I* (this is enough Bluescript for many libraries!)
 - 3 μ l pBS
 - 0.5 μ l BSA (100X stock; 0.1 mg/ml final concentration)
 - 5 μ l NEB 10X Buffer 2
 - 0.5 μ l *Xba I* (50U)
 - 41 μ l water
 - incubate at 37°C for >60'
 - Qiagen clean-up and elute in 44 μ l TE
3. CIP digest
 - add 5 μ l 10X CIP buffer directly to pBS Qiagen eluant
 - Add 1 μ l (20U) CIP
 - incubate at 50°C for 30'
 - Qiagen clean-up and elute in 30 μ l EB buffer
4. Ligation of enriched DNA to cut pBS
 - set up a 3:1 insert:vector ligation on both tetras and di's

2 μ l 10X T4 DNA ligase buffer
0.5 μ l DNA ligase (1000U)
1.0 μ l *Xba I*-cut pBS (100 ng)
1.0 μ l *Nhe I* (5U)
15.5 μ l PCR enriched genomic DNA

99 Cycles (overnight) of: 30' @ 16°C followed by 10' @ 37°C
5. Denature enzymes for 20' at 65°C and store at 4°C
6. Transform **XL2-Blue MRF'** (Stratagene #200151) competent cells (times are generally critical)
 - thaw on ice and aliquot 100 μ l cells into each of two prechilled 15-ml conical tubes
 - add 1.5 μ l **β -mercaptoethanol** to each tube and swirl on ice for 10'
 - add 1.5 μ l of each ligation (tetras and di's) to each aliquot of cells
 - ice 30'
 - heat pulse in 42°C water bath for 35"
 - ice 2'
 - add 900 μ l warm **SOC** media (no antibiotic) and incubate w/shaking 60' at 37°C
 - plate 25 μ l of each transformation on one **LB plate**, 75 μ l of each on another plate
 - incubate at 37°C overnight
 - be sure plates contain ampicillin, X-gal, and IPTG!

7. Pick white colonies and do PCR to check for inserts (optional)
 - swish each colony in 50 μ l sterile water, then inoculate 5 ml SOC & grow o/n at 37°C
 - use 4 μ l of the cell/water suspension for template in PCR
 - use T3 and T7 primers to amp inserts from white colonies (use a blue as a neg. control)
 - 4 μ l template
 - 2.5 μ l 10X *Taq* buffer
 - 1.5 μ l $MgCl_2$ (1.5 mM final concentration)
 - 4.0 μ l dNTPs (200 μ M each final concentration)
 - 0.2 μ l *Taq* (1U)
 - 0.6 μ l T3 (0.25 μ M final concentration)
 - 0.6 μ l T7 (0.25 μ M final concentration)
 - 11.55 μ l water
 - 30/30/30 @ 55°C for 32 cycles following an initial 2' denaturation at 95°C
 - Run 5 μ l PCR products on agarose gel and check for inserts

8. Screen library (optional) and sequence positive clones. After sequencing, don't forget to trim out both 1) vector sequences (e.g., pBluescript) but also 2) the SNX linker sequences that are attached to every insert!

In February of 2002, we simply sequenced the white (recombinant) colonies without any other screening. In both the salamander and kangaroo rat libraries, over 90% of the recombinant clones contained a microsatellite repeat. [This was true with both the dinucleotide enriched libraries and the tetranucleotide enriched libraries.] The efficiency was not quite as good in the otter, but even then over 60% of the di- and tetranucleotide recombinants contained microsatellites. For example, see the following table for preliminary cloning efficiencies in the kangaroo rat.

Table 1. Microsatellite clones in the kangaroo rat. Using the protocol described above, we randomly picked and sequenced three recombinants from the dinucleotide-enriched library and 22 recombinants from the tetranucleotide library. Two of the three dinucleotide clones contained microsatellites, whereas all 22 tetranucleotide clones contained microsatellites. Similar results were achieved in a salamander library, though only about 50-60% of the otter clones contained microsatellites.

Clone #	Approximate sequence length	from which library?	Repeat motif	Repeat length	Notes
A	300	di	(CT) ₂₇	54	
B	—	di	none	—	seq OK but no repeat
C	—	di	messy CT and AC's	100	near 5'
D	400	tetra	(AG) ₁₈ A(GACA) ₁₀	74	
E	250	tetra	(CTGT) ₅ (CT) ₄ (CTGT) ₂ CTTGT(CT) ₃	47	
F	350	tetra	(GA) ₃ (GACA) ₆ GCC(AG) ₅	43	
G	250	tetra	(GACA) ₇ GTCAGGCA(GACA) ₅	56	also a tetra / di repeat later
H	350	tetra	(GACA) ₆	24	
I	400	tetra	imperfect (TGTC) and (GT) ₂₄	119 and 48	
J	500	tetra	(GACA) ₈	32	
K	500	tetra	(GACA) ₇	28	
L	250	tetra	(GACA) ₇ (CA) ₆	40	
M	400	tetra	(GACA) ₈	32	also (AG) ₅
N	450	tetra	(GACA) ₉	36	near 5'
O	500	tetra	(TGTC) ₁₅	60	
P	350	tetra	(TATC) ₃ (TGTC) ₁₀ CGTC(TGTC) ₃	68	
Q	400	tetra	(GACA) ₇ (CA) ₄ AA(CA) ₈	54	
R	450	tetra	(GACA) ₁₂	48	another compound at 400 bp
S	350	tetra	(GACA) ₈	32	
T	200	tetra	(GACA) ₈	32	
U	400	tetra	(TGTC) ₁₂	48	also (TG) ₂₀
V	200	tetra	messy GT GA & GACA		
W	350	tetra	(TG) ₁₉ (TGTC) ₈	70	
X	400	tetra	(TGTC) ₈ (TATC) ₆	56	
Y	400	tetra	(TGTC) ₇	28	