[13] Isolating Microsatellite DNA Loci

By TRAVIS C. GLENN and NANCY A. SCHABLE

Abstract

A series of techniques are presented to construct genomic DNA libraries highly enriched for microsatellite DNA loci. The individual techniques used here derive from several published protocols but have been optimized and tested in our research laboratories as well as in classroom settings at the University of South Carolina and University of Georgia, with students achieving nearly 100% success. Reducing the number of manipulations involved has been a key to success, decreasing both the failure rate and the time necessary to isolate loci of interest. In our lab during the past 3 years alone, these protocols have been successfully used to isolate microsatellite DNA loci from at least 55 species representing three kingdoms. These protocols have made it possible to reduce the time to identify candidate loci for primer development from most eukaryotic species to as little as 1 week.

Introduction

Microsatellite DNA loci have become important sources of genetic information for a variety of purposes (Goldstein and Schlotterer, 1999 see Chapter 14). To amplify microsatellite loci by polymerase chain reaction (PCR), primers must be developed from the DNA that flanks specific microsatellite repeats. These regions of DNA are among the most variable in the genome, thus primer-binding sites are not well conserved among distantly related species (Moore *et al.*, 1991; Pepin *et al.*, 1995; Primmer *et al.*, 1996; Zhu *et al.*, 2000). Although microsatellite loci have now been developed for hundreds of species (indeed the journal *Molecular Ecology Notes* is largely devoted to their description), these loci have not been isolated from many additional species of interest and remain to be developed.

Many strategies for obtaining microsatellite DNA loci have been described. The simplest approach, cloning small genomic fragments and using radiolabeled oligonucleotide probes of microsatellite repeats to identify clones with microsatellites, was the first described and works well in organisms with abundant microsatellite loci (Tautz, 1989; Weber and May, 1989; Weissenbach *et al.*, 1992). Unfortunately, this approach does not work well when microsatellite repeats are less abundant. Thus, two classes of enrichment strategies have been developed: (1) uracil-DNA selection

0076-6879/05 $35.00

(Ostrander *et al*., 1992) and (2) hybridization capture (Armour *et al*., 1994; Kandpal *et al*., 1994; Kijas *et al*., 1994). Hybridization capture is the predominant strategy in use because it allows selection before cloning and, thus, is faster and easier to do with multiple samples than uracil-DNA selection, which requires passage of each library through two bacterial strains.

We have refined the hybridization capture approach described by Hamilton *et al.* (1999), which derives from Armour *et al.* (1994), Fisher and Bachmann (1998), Kandpal *et al.* (1994), Kijas *et al.* (1994), and others (Zane *et al*., 2002). In theory, this protocol will work for any eukaryotic organism (i.e., anything with an appreciable number of microsatellite loci) or any other piece of DNA that may be captured using an oligonucleotide. The biggest differences among DNA samples (i.e., species from which the DNA derives) are (1) how the initial DNA sample is isolated and (2) which microsatellite repeats occur most frequently in a particular organism and, thus, are targeted for enrichment and isolation. In practice, we and our collaborators have used this protocol to construct libraries and determine flanking sequences of microsatellite DNA loci in amphibians, birds, fish, mammals, reptiles, insects, nematodes, and various other invertebrates, fungi, plants, and coral. The protocol has been outlined in several publications (Hauswaldt and Glenn, 2003; Korfanta *et al*., 2002; Prince *et al*., 2002; Schable *et al*., 2002) but has many fine points that are not likely to be obvious from those publications.

The most unique feature in this protocol is the incorporation of a GTTT ''pig tail'' on the SuperSNX linker and modification of the originally described SNX sequence. The SuperSNX linker has PCR characteristics even better than the SNX linker in the study by Hamilton *et al.* (1999) and ensures efficient A-tailing of each PCR product yielding good results from TA cloning. Interestingly and most importantly, amplification of DNA using the SuperSNX linker/primer is biased against producing small PCR products. Thus, PCR products obtained after enrichment can be cloned directly without obtaining a large proportion of small DNA fragments. Most of the other details in this protocol have been reported previously or have been generously provided by colleagues; we have simply compiled the best specific approaches from many protocols to reduce the time and steps required to isolate microsatellite DNA loci.

Step I: Extracting DNA

Goal: To Isolate about 10 μg of high-molecular-weight DNA (ideally 50^{+} μl of 100^{+} ng/μl). About 2–3 μg of good DNA will suffice and considerably less can be used, but it is a good idea to have much more than minimal amounts of DNA available. For most organisms, it is best to perform a PCI extraction, followed by an ethanol precipitation

(Sambrook *et al*., 1989). Many people also have very good success with silica-based protocols such as DNeasy kits (Qiagen, Valencia, CA), Wizard Preps (Promega Corp., Madison, WI), or homemade equivalents (http://www.uga.edu/srel/DNA_Lab/MUD_DNA′00.rtf_.rtf). The only caution offered regarding the use of such protocols is that the recovered concentration of DNA is usually 50–100 ng/μl and is often less than that. Thus, you may need to do an ethanol precipitation of the DNA recovered from a Qiagen kit (or comparable kits or method used) to increase the concentration of DNA. It is also best to destroy the RNA by performing the ''optional'' RNase treatment during the DNA extraction.

Detailed steps: It is imperative to check the concentration and quality of the DNA before proceeding.

1. Quantify the DNA concentration and examine its quality by diluting 2 μl of DNA with 3 μL of TLE (10 m*M* Tris, pH 8.0, 0.2 m*M* EDTA), 2 μl of loading buffer (Sambrook and Russell, 2001; Sambrook *et al*., 1989), and loading onto a 1% agarose gel containing ethidium bromide. Use 50 and 200 ng of uncut lambda DNA as standards. DNA quantity must be at least as bright as the 50-ng band and ideally as bright as the 200-ng band of lambda DNA. DNA quality is assessed by the absence (high quality) or presence (lower quality) of a smear down the gel when compared to lambda DNA. Any remaining RNA will also appear as a smear much smaller than the lambda DNA.

Choosing DNA for Marker Development

It is best to use DNA of the highest quality that can be reasonably obtained. In practice, most projects start with fewer than 10 DNA samples, and the best 2 among those are used. A small to modest amount of DNA smearing down below 5000 base pairs (bp) is generally fine. If a substantial proportion of the DNA is less than 5000 bp (especially if $<$2000 bp), then it will be worthwhile to do additional DNA extractions to obtain higher quality DNA.

There are some advantages and some disadvantages to mixing DNA from multiple individuals. In general, we recommend using DNA from one individual (heterogametic sex if there is an interest in possibly obtaining a sex-specific marker) of one species. The use of markers from any one individual may create an ascertainment bias (especially when the markers developed are applied to other species), but at least the researcher will be aware of the potential bias. If a mixture of DNA from different individuals is used, then it may never be known from which individual any particular clone (locus) came. Subsequently, it may be difficult or impossible to

resolve the source of problems that may be encountered further in the development process as a result of using multiple DNAs. If one would like to use the markers for multiple species for species that are closely related, then development of one library will likely be sufficient (all else being equal, it may be best to choose the basal species). Another approach is to develop loci from two species—ideally the two least related (i.e., most distant phylogenetically).

Step II: Restriction Enzyme Digest

Goal: To fragment the DNA into approximately 500 bp fragments. After several steps, these fragments will be inserted into a plasmid and then bacteria. Fragments of this size are small enough to sequence easily while retaining a high probability of having enough DNA flanking the microsatellites that primers can be designed. Restriction enzymes are an easy way to fragment the DNA. Restriction enzymes recognize specific sequences and will cut the DNA at this site, leaving a known end that will prove helpful later in this protocol. The following restriction enzymes have been used: *Rsa*I and *Bst*UI. These restriction enzymes can be purchased through New England BioLabs (NEB). To learn more about these or other enzymes, NEB has an informative web site (http://www.neb.com). Any frequent cutting restriction enzyme that leaves a blunt end could be used, although it is best if they are heat labile and work in NEB buffer no. 2.

It is best to begin by setting up a digest on two DNA samples using *Rsa*I. If the resulting smear is not continuous with most of the DNA ranging from approximately 300 to 1000 bp, then it is wise to attempt another digest on uncut DNA using *Bst*UI.

Recipe

*Rsa*I or *Bst*UI[1]

2.50 μl NEB 10$\times$ ligase buffer (*note:* heat to 50° or 65° to get all components in solution)

[1] *Rsa*I recognizes GT^AC and *Bst*UI recognizes CG^CG; so one may work better than another in any particular organism. *Hae*III (GG^CC) could also be used, but it has a recognition site in SuperSNX. Linker ligations are still generally successful on DNAs digested using *Hae*III because only a small proportion of the linker is digested in the subsequent linker ligation reaction. Using *Hae*III is less than optimal, so its use is not recommended unless other restriction enzymes have failed to yield DNAs cut to an average of about 500 bp in length. If these enzymes result in fragments that are too small, 6-base cutting alternatives include the following: *Eco*R V (GAT^ATC; only 75% efficient in this buffer but still adequate; (*Ssp*I) (AAT^ATT), *Stu*I (AGG^CCT), and *Sfo*I (GGC^GCC).

0.25 μl 100× bovine serum albumin (BSA) (BSA supplied with enzymes from NEB)

0.25 μl 5 *M* NaCl (50 m*M* final)

1.00 μl *Rsa*I (NEB catalog no. R0167S) or *Bst*UI (NEB catalog no. R0518S)

1.00 μl XmnI (NEB catalog no. R0194S; *note:* XmnI can be added at step III.2, later in this chapter instead)

20.0 μl[2] genomic DNA (100 ng/ μL).

If plenty of DNA is available and saving time is important, then one may perform digests with each enzyme (in separate tubes) simultaneously. Because some enzymes may give biased results, it is potentially helpful to combine ligations from multiple enzymes. Note that it is unwise to cut the DNA with multiple 4-base cutting enzymes at once or to combine the DNA until after the linker ligation. If digests are combined before the linkers are ligated, then one will not be able to determine whether multiple unrelated DNA fragments have been joined (i.e., ligated into chimeras), potentially resulting in unamplifiable loci.

Detailed steps:

1. To make master mixes for *Rsa* I and *Bst*U I, multiply the volume of each of the components in the preceding list by the number of DNA samples to be digested, plus half a sample to account for pipetting error and add to a 1.5-ml tube.
2. Prepare the restriction enzyme digest for *Rsa* I and *Bst*U I by adding 5 μl of master mix into a new tube (0.2 or 0.5 ml depending on thermal cycler available) and use a thermal cycler for all incubations. Add 20 μl of DNA to each tube. Pipette up and down to mix the solution.
3. Incubate all samples (*Rsa* I and *Bst*U I) at 37° for 30–60 min.
4. While the restriction digest is incubating, pour a 1% agarose gel, including ethidium bromide (Sambrook *et al*., 1989).
5. Set aside a small aliquot (4 μl) of the digested DNA.
6. Immediately proceed to step III.

Note: You will run the aliquot of the restriction enzyme digest on a 1% agarose gel to verify that the restriction enzyme digest was successful at step III.4 below.

[2] Assumes a DNA concentration of about 100 ng/μl (i.e., ~2 μg of DNA). Adjust accordingly if the DNA is significantly more concentrated (i.e., if >200 ng/μl, use less and make up the volume in water). This recipe may still be used if less than 20 μl of 100 ng/μl of DNA is available, but it may be necessary to amplify the DNA with the SuperSNX24 primer before enrichment (especially if the amount of DNA available is <100 ng). It is important to note that amplifying the linker ligated DNA before enrichment may bias the enrichment results.

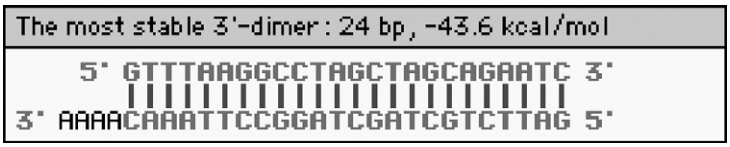
Step III: Ligating Linkers to DNA Fragments

Goal: To ligate a double-stranded linker onto both ends of each DNA fragment. The linkers will provide the primer-binding site for subsequent PCR steps. They also provide sites to ease cloning of the fragments into the vectors that will subsequently be used. The linkers are, therefore, compatible with the restriction sites in the vector's multiple cloning site. The SuperSNX also incorporates a GTTT ''pigtail'' to facilitate nontemplate A addition by *Taq* DNA polymerase during PCR, which can be used for TA cloning.

Note: This protocol is written with the assumption that there is only a need for two enrichments per linker-ligated DNA. There is enough linker ligation for three enrichments using the recipes below. If one plans to perform more than three enrichments, it is important to scale up reaction volumes, set up additional linker ligations, decrease the amount of linker-ligated DNA used in step IV or use PCR-amplified linker-ligated DNA (from step III.6).

SuperSNX24 Forward: 5′GTTTAAGGCCTAGCTAGCAGAATC

SuperSNX24 + 4P Reverse: 5′pGATTCTGCTAGCTAGGCCTTAAA CAAAA



Note: The phosphate (p) on SuperSNX24 + 4P Reverse allows ligation of the linkers to each other or the digested DNA, but it is not shown in the Fig.

Detailed steps:

1. Preparation of double-stranded (ds) SuperSNX linkers:
   Mix equal volumes of equal molar amounts of SuperSNX24 and SuperSNX24 + 4p primers (e.g., 100 μl of 10 μM each). Add salt to a final concentration of 100 mM (i.e., 4 μl of 5 M NaCl for 200 μl of primers). Heat this mixture to 95°, and let it cool slowly to room temperature to form the ds SuperSNX linkers.
2. Linker-ligation recipe: (*note:* add Xmn I if it was not added above]:
   7.0 μl ds SuperSNX linkers
   1.0 μl 10× ligase buffer (ensure components are in solution—warm if necessary)
   2.0 μl DNA ligase (NEB #M0202S; 400 units/μl)

   10.0 μl total

If multiple DNAs are being ligated, make a master mix of the components listed above the line and add them (10 μl of mix) to the cut DNAs.

3. Incubate at room temperature for 2 or more h or ideally at 16° overnight.

4. While the ligation is proceeding, run the small aliquots of restriction enzyme–digested DNA (from step II.5) on the 1% minigel (from step II.4) to ensure the DNA samples were successfully digested. A successful reaction should yield a smear of fragments centered at approximately 500 bp, but at least with most fragments at or below 1000 bp.

5. To ensure ligation was successful, perform a PCR on the linker ligation using the following recipe for a 25-μl reaction (a 50-μl reactions volume should be used when enrichment will be performed using the PCR products):

2.5 μl 10× PCR buffer (optimal buffer for *Taq* used below)
2.5 μl BSA (250 μg/ml → 25 μg/ml final)
1.3 μl SuperSNX24 (10 μM → 0.5 μM final)
1.5 μl deoxyribonucleic triphosphates (dNTPs) (2.5 mM each → 150 μM final)
2.0 μl $MgCl_2$ (25 mM → 2.0 mM final)
13.0 μl dH_2O
0.2 μl *Taq* DNA polymerase (5 units/μl)

2.0 μl linker-ligated DNA fragments

If multiple DNAs are being tested, make a master mix of the components above the solid line and add them (23 μl of mix) to the linker-ligated DNAs (2 μl).

Note: Only one primer is used, see note V. 1.

Cycling: 95° for 2 min; then 20 cycles at 95° for 20 s, 60° for 20 s, 72° for 1.5 min. Hold at 15°.

Note: The same program as the enrichment recovery (step V) may be used, but it takes longer.

6. Run 4 μl of PCR product on a 1.0% minigel to see if the linker ligation was successful using a 100-bp ladder as a size standard. A successful reaction should yield a smear of fragments centered at approximately 500 bp. This PCR product can be used for enrichment if insufficient amounts of original linker-ligated DNA are available.

Notes:

- All restriction enzymes must be kept on ice until use and immediately placed back in the −20° freezer after use.
- Three to five times the amount of linkers relative to each fragment increases the odds that the linkers will ligate to a DNA fragment instead of the latter to each other.

- *Conversion factors:* 1 μg of 1-kb fragments = 3.3 pmol fragment ends. Also, 1 μl of X μM linkers = X pmol of linker ends.
- The *Xmn I* prevents the dimerization (self-ligation) of linkers, *so it is vital for success*.

Step IV: Dynabead Enrichment for Microsatellite-Containing DNA Fragments

Goal: To capture DNA fragments with microsatellite sequences complementary to the microsatellite oligos (probes) and wash away all other DNA fragments.

Note: This protocol is written with the assumption that there is a need for only two enrichments per linker-ligated DNA (see note III.1 above). If one plans to perform serial (double) enrichments, it is a good idea to set up replicate enrichments.

Materials and Solutions

Washed Dynabeads (see step 7—wash twice in TE [10 mM Tris pH 8.0, 2 mM EDTA] and twice in 1× Hyb solution) *note:* each 50 μl of Dynabeads (Dynal, Oslo, Norway) can capture 100 pmol of biotinylated oligo. It is critical to have an excess of bead capacity relative to the amount of biotin/oligo added. If beads from other manufacturers are used, the amount of beads should be adjusted to account for variation in biotin-binding capacity.

2× Hyb Solution: 12× SSC, 0.2% SDS (warmed; stock solution 20× SSC: 3.0 M NaCl, 0.3 M sodium citrate, pH 7.0).

1× Hyb Solution: 6× SSC, 0.1% SDS (warmed to get everything into solution).

Washing Solutions: 2× SSC, 0.1% SDS (warmed to get everything into solution), 1× SSC, 0.1% SDS (warmed to get everything into solution).

Biotinylated oligos: Mixtures of 3′ biotinylated oligos are used with this protocol (see http://www.uga.edu/srel/Msat_Devmt/Probe_List.htm) (note there are underscores [not spaces] in that web address). 3′ labeling is used because it has the highest efficiency of labeling (each oligo synthesis starts with a biotin). A large number of oligos may be used in a mix together when their lengths are varied to achieve similar melting temperatures (T_ms). We use oligos purified by standard desalting methods (i.e., no additional purification by high-performance liquid chromatography [HPLC], gels, etc.), because we order large numbers

of oligos and the additional purification would be quite expensive. The critical factor to keep in mind when using biotinylated oligos purified by standard desalting methods is that the solution will contain many "free" biotins, so it is critical to ensure the amount of biotin (estimated from the oligo concentration) added is *far* less than the bead-binding capacity.

NaOAc EDTA Solution: To a 50-ml conical, make 20 ml of 3 *M* NaOAc from the dry chemical stock. Do not adjust the pH. Add 20 ml of 500 m*M* EDTA, pH 8.0. This makes a solution that is 1.5 *M* NaOAc and 250 m*M* EDTA. Aliquot into 1.5-ml microcentrifuge tubes and/or 0.2-ml strip tubes and freeze.

Detailed Steps

1. In a 0.2-ml PCR tube, add:
25.0 μl 2× Hyb solution (warmed to get everything into solution)
10.0 μl biotinylated microsatellite probe (mix of oligos at 1 μM each)
10.0 μl linker-ligated DNA from step III (or PCR product if <2 μg DNA initially used)
5.0 μl dH_2O

50.0 μl Total

2. Use thermal cycler program *OligoHyb*. This program denatures the DNA–probe mixture at 95° for 5 min. It then quickly ramps to 70° and steps down 0.2° every 5 s for 99 cycles (i.e., 70° for 5 s, 69.8° for 5 s, 69.6° for 5 s, ... down to 50.2°), and stays at 50° for 10 min. It then ramps down 0.5° every 5 s for 20 cycles (i.e., 50° for 5 s, 49.5° for 5 s, 49° for 5 s, ... down to 40°), and finally quickly ramps down to 15°. The idea is to denature everything, quickly go to a temperature slightly above the annealing temperatures of the oligos in the mixes used, and then slowly decrease, allowing the oligos the opportunity to hybridize with DNA fragments that they most closely match (i.e., hopefully, long perfect repeats) when the solution is at or near the oligo's T_m.

3. While the DNA–probe mixture is in the thermal cycler, wash 50 μl of Dynabeads (Dynal, Oslo, Norway). Resuspend the beads in their original tube, and transfer to a 1.5-ml tube. Add 250 μl of TE. Shake. Capture beads using the Magnetic Particle Concentrator (MPC) (Dynal, Oslo, Norway). Repeat with TE, and twice with 1× Hyb solution. Resuspend the final beads in 150 μl of 1× Hyb solution.

4. Pulse-spin your DNA–probe mix and add all of it to the 150 μl of washed, resuspended Dynabeads (i.e., to the 1.5-ml tube).

5. Incubate on rotator or sideways in orbital shaker on slow speed at room temperature for 30 or more min.

6. Capture beads using the MPC. Remove the supernatant by pipetting with a P200 pipetter (*Optional:* Save supernatant for troubleshooting purposes).

7. Wash the Dynabeads two times with 400 μl 2$\times$ SSC, 0.1% SDS each time using the MPC to collect the beads and removing the supernatant by pipetting with a P200 pipetter (which can be saved for troubleshooting purposes). Resuspend beads well (i.e., flick or gently vortex) in next wash each time.

8. Wash two additional times using 400 μl 1$\times$ SSC, 0.1% SDS.

9. Wash two final times using 400 μl 1$\times$ SSC, 0.1% SDS, and heating the solution to within 5–10° of the T_m for the oligo mix used (usually 45 or 50°). *Note:* More stringent washes may increase the relative proportion of long microsatellite sequences in cloned colonies, but they may also cause the loss of microsatellite sequences via undesired elution during washing.

10. Add 200 μl TLE, vortex, and incubate at 95° for 5 min. Label a new tube while incubating. Capture beads using the MPC. Quickly remove the supernatant by pipetting to the new tube. This supernatant contains the enriched fragments (i.e., ''the gold'').

Note: It is important to remove the supernatant from the beads reasonably quickly after removing from the 95° heat block. It is not unusual for the supernatant to have slight discoloration from the beads (appears that the magnet is not working well). A *very small* amount of discoloration (leading to a colored pellet following precipitation) does not seem to be harmful.

11. Add 22 μl of NaOAc/EDTA solution (see ''Recipe'' above). Mix by pipetting up and down.

12. Add 444 μl of 95% EtOH. Mix by inverting the tube and place on ice for 15 min or more (or store in the −20° freezer for as long as desired).

13. Centrifuge at full speed for 10 min.

14. Discard supernatant and add approximately 0.5 ml of 70% EtOH. Centrifuge for 1 min.

15. Carefully pipette off *all* the supernatant and air-dry the sample. If there is any visible trace of EtOH, pulse-spin the tube and use a pipette to remove any residual EtOH. Dry until there is no trace (smell) of EtOH.

16. Resuspend the pellet in 25 μl of TLE. This is the ''pure gold.'' Let the pellet hydrate while setting up PCRs in step V (at least 20 min). It may be best to allow for overnight rehydration to be sure that the DNA is in solution. Inadequate rehydration is the most common reason for failure of the next step.

Step V: PCR Recovery of Enriched DNA

Goal: To increase the amount of ''pure gold'' DNA. To do serial (double) enrichments, use the resulting PCR products (step V.2) for the second enrichment.

Detailed steps:

1. Perform PCR on supernatant (step IV.16) to recover the enriched DNA fragments:

2.5 μl 10$\times$ PCR buffer (optimal buffer for *Taq* used below)
2.5 μl BSA (250 μg/ml $\rightarrow$ 25 μg/ml final)
1.5 μl dNTPs (2.5 m*M* each $\rightarrow$ 150 μ*M* final)
1.3 μl SuperSNX-24 (10μ*M* $\rightarrow$ 0.5 μ*M* final)
2.0 μl $MgCl_2$ (25 m*M* $\rightarrow$ 2.0 m*M* final)
13.0 μl dH_2O
0.2 μl *Taq* DNA polymerase (5 units/μl)

2.0 μl eluted DNA fragments (''pure gold'') (*note:* Ensure gold pellet has hydrated for 20 min or longer—longer is better; lots of mixing ensures the pellet becomes rehydrated and the PCR will be successful)

If multiple DNAs are being tested, make a master mix of the components above the solid line and add them (23 μl of mix) to the eluted DNAs (2 μl). It is often wise to perform a second PCR, using half as much eluted DNA (i.e., 1.0 μl of eluted DNA + 1.0 μl of dH_2O).

Cycling: 95° for 2 min; then 25 cycles of 95° for 20 s, 60° for 20 s, 72° for 1.5 min; then 72° for 30 min; then hold at 15°.

Note: It is correct that only one primer is used (the SuperSNX24 Forward primer). If both forward and reverse primers are used, then the reaction will fail. If one draws an example of the linker-ligated DNA in double-stranded form, it is easier to visualize why using only one primer works.

5′ForwardPrimer::DNAofInterest::ReversePrimer3′

3′ReversePrimer::DNAofInterest::ForwardPrimer5′

2. Run 4 μl of PCR product on a 1.0% minigel next to a 100-bp ladder as a standard to verify whether DNA recovery was successful. *Note:* The smear of fragments should be visible, centered at approximately 500 bp. If bands are visible, it is likely few microsatellite loci are present; setting up multiple PCRs may increase the chances that one of the reactions will not have defined bands. If setting up multiple PCRs fails to yield bandless smears of product, enrich using a different mix of oligos.

Optional: For troubleshooting and to verify enrichment success, use 2 μl of PCR product for a dot-blot analysis (see step XII of *Msat_Easy_Isolation _2000.rtf* available at http://www.uga.edu/srel/DNA_Lab/protocols.htm)

to ensure that microsatellite containing fragments have been recovered. Use genomic DNA and PCR product from the linker-ligation check as rough controls (the enriched PCR product resulting dot should be much darker than either the genomic DNA or the linker-ligation check PCR dots). For the best comparison, one should add an equal number of nanograms of DNA from the enriched PCR and the linker-ligation check PCR. Diluting each PCR product a few times (e.g., five, ten, or fifty times) will most likely enhance the ability to see differences among dots. The leftover linker-ligated DNA includes fragments that are too long to be recovered by PCR (thus, biasing the comparison somewhat). If the linker-ligation check PCR dot is not very dark and the linker-ligated DNA is very dark, it may be wise to use a different mix of microsatellite oligos or to use a different restriction enzyme for the linker ligation.

3. Make S-Gal Amp or LB Amp plates in preparation for transformation (transformation step 2 below). Four or more S-Gal Amp or LB Amp (50–100 μg/ml) bacterial plates will be needed for each successfully enriched PCR. Follow the protocol from Sigma for S-Gal, in the Invitrogen Topo-TA manual, or Sambrook *et al*. (1989).

Step VI: Ligating-Enriched DNA into Plasmids

Goal: To incorporate (ligate) the enriched/ recovered DNA (amplified pure gold) into a cloning vector. The idea is to place one fragment of the DNA into one vector and to do this for as many fragments as possible. Once ligated into the vector, the DNA is known as an *insert*.

This protocol assumes that it is best to use the fastest and most reliable method available. In our experience and the experience of our colleagues, the TA cloning kits from Invitrogen (Carlsbad, CA) are the quickest and most robust. It is important to point out, however, that there is nothing wrong with using TA cloning kits from other vendors (e.g., Promega, Madison, WI) or homemade preps (Holton and Graham, 1991; Marchuk *et al*., 1991). Both the TOPO TA Cloning Kit containing pCR 2.1-TOPO with TOP 10 cells (catalog K4500-40) as well as the kit with TOP 10F′ cells (catalog K4550-40) have successfully been used with this protocol. In general, the former option is recommended because it does not require the use of IPTG. If only a small number of ligations and transformations will be performed, one can purchase the 20 reaction kit (K4500-01), rather than the 40 reaction kit.

Detailed steps:

1. Follow the directions supplied with Invitrogen's TOPO-TA cloning kit *exactly*! If another TA cloning protocol is used, follow the appropriate directions for ligation and transformation.

2. Warm S-Gal/LB Agar (Sigma) Amp plates before starting transformation (LB Amp plates spread with X-Gal and IPTG may be substituted).

Note: It is important to know that the restriction sites in the SuperSNX linker can be used for sticky-end cloning, exactly as described by Hamilton *et al*. (1999). If many enrichments will be performed, it may be wise to invest the time to use that approach, which is a superior method in many ways. The major disadvantage is that the enriched DNA must be cleaned after digestion of the linker end, which requires additional time and more steps (increasing the likelihood of the product getting lost or having other ''handling tragedies,'' especially in the hands of inexperienced workers). In addition, some DNA fragments will contain *Nhe*I restriction sites, and thus, the number of inserts with no microsatellites and those with little flanking DNA will be increased (this can be countered to some extent by adding *Nhe*I to the original restriction enzyme digest—step II).

Step VII: Transforming Plasmid DNA

Goal: To incorporate your enriched/recovered DNA (amplified pure gold; or insert) + cloning vector into a bacterial host. The idea is to place one vector (which, ideally, has one fragment of amplified pure gold [insert]) into one bacterial host, and do this for as many vectors–inserts as possible. Usually ampicillin (*amp*)-sensitive bacteria and a vector that carries a gene conferring *amp* resistance are used. When a bacterium incorporates the vector, the vector transforms the phenotype of the bacterium from *amp* sensitive to *amp* resistant. Thus, when a mixture of bacteria is plated on media containing *amp*, only bacteria with *amp* resistance (i.e., those that have incorporated the vector) can grow and form colonies. *Note:* This step continues with the assumption that the TA cloning kits from Invitrogen are used. Other standard transformation protocols are available (Sambrook and Russell, 2001; Sambrook *et al*., 1989).

Detailed steps:

1. Follow Invitrogen's TOPO-TA cloning kit *exactly*!
2. Following the 1-h incubation in SOC, plate out 25 μl of transformed bacteria onto two plates and 50 μl of transformed bacteria onto two other plates. This will ensure that plenty of colonies will be present, but that they are not growing on top of each other. This will probably yield enough colonies, but it is reasonable to plate out the entire amount, or one may save the remaining transformed bacteria in broth (at 4°) to be plated the next day (waiting to plate is particularly valuable if one is unsure of the quality of the plates or is trying to minimize the number of plates used;

the number of colonies obtained per microliter will, however, be reduced by plating at a later time).

3. Grow colonies overnight at 37°.

Step VIII: PCR and Storing Positive Colonies

Goal: To determine the number and proportion of colonies with vector and vector–insert, to amplify inserts from the bacteria/vectors, and to archive bacteria from each colony of interest (i.e., those with inserts).

Detailed steps:

Day 1

1. Count the number of blue (or black, if S-Gal was used) colonies and the number of positive (white) colonies on each plate. If more than a few hundred are present, simply note that fact rather than trying to count each colony. The proportion of colonies with inserts (i.e., vector ligation efficiency) can be determined from the number of white colonies divided by the total number of colonies.

2. Prepare 50 ml of LB broth with ampicillin by adding 50 μl of ampicillin (50 mg/ml stock) to a 50-ml conical tube full of broth. Add 300 μl of LB broth + ampicillin to each well of a sterilized 0.65 ml deep-well plate.

3. Lift isolated white colonies from the LB plate using the end of a sterile toothpick and transfer each colony to one well of the sterilized deep-well plate (spin the toothpick in your fingers while the end with the colony is immersed in the LB broth).

4. Cover the 96 deep-well plate loosely with Saran Wrap or a loose-fitting 96-well mat. Incubate overnight at 37° with semivigorous shaking. It is often beneficial to incubate an additional 24 h (~40 h total) to achieve high-density cell growth.

Day 2

5. Set up the following 25-μl PCRs:

For one 96-well tray, add the following to a clean V-bottom trough:

275.00 μl	250 μg/ml BSA
275.00 μl	10× PCR buffer
110.00 μl	10 μM M13 forward primer
110.00 μl	10 μM M13 reverse primer
220.00 μl	25 mM $MgCl_2$
165.00 μl	2.5mM dNTPs (2.5 mM each)
1408.00 μl	dH_2O
22.00 μl	*Taq* DNA polymerase (2.5 units/μl)

Using a multichannel pipetter, dispense 23.5 μl to each well of 96-well thermal plate, and then add 1.5 μl DNA template from bacteria colony grown up in LB broth. If setting up fewer than 96 reactions, the 25-μl reaction recipe (per reaction) is as follows:

2.50 μl	250 μg/ml BSA
2.50 μl	10x PCR buffer
1.00 μl	10 μM M13 forward primer
1.00 μl	10 μM M13 reverse primer
2.00 μl	25 mM $MgCl_2$
1.50 μl	2.5 mM dNTPs (2.5 mM each)
12.80 μl	dH_2O
0.20 μl	*Taq* DNA polymerase (2.5 units/μl)
1.50 μl	DNA template from bacteria colony grown up in LB broth

Cover the reactions using a mat or caps and place the PCRs in the thermal cycler. Store bacteria colonies in LB broth at 4° until PCR product has been observed.

Cycling: 95° for 3 min; then 35 cycles of 95° for 20 s, 50° for 20 s, 72° for 1.5 min. Hold at 15°.

After the PCR is finished, the product will need to be examined for the presence of inserts in each plasmid.

6. Pour a 1% agarose gel on the centipede rig (Owl Scientific, Portsmouth, NH).

7. Run 2 μl of the M13/bacterial PCR product on the agarose gel along with a 100-bp ladder and 2 μl of several lambda concentration standards (λ10 ng/μl, λ25 ng/μl, λ50 ng/μl, and λ100 ng/μl). A 10-μl multichannel pipetter may be used to save time. It loads every other lane, so it is important to keep notes on where each sample is located.

8. Run at 80 V for approximately 30–40 min.

9. Examine PCR results using a visual imaging system and save the results. Ensure that bands are clearly visible, but that they are not saturated/overintegrated (red). If DNA concentration varies a lot (i.e., the brightest samples are red when you are exposing enough to see the dimmest samples, then save multiple exposures).

10. The desired insert range is from 300 to 1000 bp. Because the pCR2.1 vector contains about 200 bp of DNA between the M13 forward and reverse priming sites, the total fragment size of desired PCR products is 500–1200 bp. Proceed to purification step using only samples that are the target size.

After the bacteria have grown overnight and the PCRs have been examined, perform the following:

11. To a 50-ml conical tube, add 15 ml of glycerol, fill to 50 ml with LB broth, and add 50 μl of ampicillin (50 mg/ml stock). Mix thoroughly by shaking.
12. Remove the bacterial cultures from the refrigerator or incubator.
13. Using the multichannel pipette, add 300 μl of prepared broth to each culture (being careful not to contaminate samples), tightly seal the cap mat on the tray, mix gently by inverting several times, label well, and store at -70°.

Step IX: Prepare PCR samples for Sequencing using ExoSAP

Goal: To determine PCR product concentration and size and to purify the PCR product for subsequent sequencing. There are many ways to prepare PCR products for sequencing. If the PCR products are good and strong, dilution (i.e., using no more than 0.5 μl of PCR product) is efficient and usually works well. However, cleaning the PCR using exonuclease I and SAP is a preferred option, which improves consistency among experiments and researchers in our lab.

Materials and Solutions

Premixed ExoSAP: catalog no. 78201, U.S. Biochemical Corp., Cleveland, OH, or Homemade ExoSAP: combine 5 μl of 20 units/μl exonuclease I (NEB catalog no. M0293L) with 15 μl of 1 unit/μl SAP (U.S. Biochemical Corp., catalog no. 70092Z).

1. Quantify PCR product concentration and size. For a single sequencing reaction, the desired amount of template is 10 ng of PCR product per 100 bp of length (i.e., for a 500-bp product, 50 ng is needed). Generally, we purify enough PCR product for two sequencing reactions. Use the visual imaging results saved from step VIII.9 above.
2. Add 1 μl ExoSAP mixture to 6–10 μl of PCR product. If PCR products are of varying concentration, the concentrations can be standardized by adding H_2O (*note:* Volumes should be adjusted to account for differences in lengths of the PCR products so that the molar concentrations are approximately equal).
3. Incubate the samples at 37° for 15 min, 80° for 15 min, and then hold at 15°. The samples are now purified and ready for use as sequencing reaction template.

Step X: DNA Cycle Sequencing Reactions

Goal: To complete sequencing reactions that can be used to determine the DNA sequence of the fragments that contain microsatellite repeats.

This protocol is optimized for 0.2-ml strip tubes and a titer plate centrifuge but can be used with 96-well plates with only minor modifications.

Alternative 1[3]: One-Fourth Reaction Recipe

2.0 μl BigDye Terminator version 3.1 mix[3]
1.0 μl 5× Sequencing Dilution Buffer[4]
1.0 μl primer[5](3.3 μM)

Note: Make a master mix of the above for the number of reactions being set up. If the template concentration is constant, water may also be added into the master mix.

2.0 μl DNA template (10 ng/ 100 bp of product length; adjust volume as appropriate)[6]
4.0 μl H_2O (adjust volume as appropriate to make a total of 10.0 μl)

Alternative 2[3]: One-Eighth Reaction Recipe

1.0 μl BigDye Terminator version 3.1 mix[3]
1.5 μl 5× Sequencing Dilution Buffer[4]
1.0 μl Primer[5] (3.3 μM)

Note: Make a master mix of the above for the number of reactions being set up. If the template concentration is constant, water may also be added into the master mix

2.0 μl DNA template (10 ng/100 bp of product length; adjust volume as appropriate)[6]
4.5 μl H_2O (adjust volume as appropriate to make a total of 10.0 μl)
Cycling: 50 cycles at 96° for 10 s, 50° for 5 s, 60° for 4 min. Hold at 15°.
Note: No initial denaturation is necessary.

[3] Depending on level of experience, instruments available, and success, one may want to use standard, half, quarter, one-eighth, one-twelfth, or one-sixteenth reactions. Adjust BigDye and dilution buffer appropriately (*note:* the BigDye is at 2.5× and dilution buffer is at 5×).
[4] A homemade version of Sequencing Dilution Buffer is 400 mM Tris–HCl, pH 9.0; 10 mM $MgCl_2$ (see http://www.genome.ou.edu/proto.html for details), which we have used successfully as a 5× or 2.5× buffer.
[5] M13 forward or reverse, or other primers closer to the insertion site, as appropriate.
[6] If templates are consistent in size and concentration, water may be combined in the master mix and a constant volume of template may be used.

Place the tubes in a 96-well holder and store the reactions at −20° in a *non*–frost-free freezer until they can be precipitated. The reactions are stable for days at this stage, but it is best to keep them cold or frozen and away from light.

Step XI: Precipitation of Sequencing Reactions

Goal: To remove the unincorporated fluorescent ddNTPs and stabilize the labeled DNA until it can be run on an automated DNA sequencer. There are several options for cleaning sequencing reactions. Column purification (Sephadex G50/ Centri-Sep columns from Princeton Separations, Adelphia, NJ) is often superior in that bases close to the primer are more likely to be recovered and the remaining salts are reduced, which is best when using capillary sequencers. The ABI BigDye version 3.1 manual includes a protocol very similar to the one below, except that NaOAc is not used. We use the following protocol with good success on ABI 377, 3700, and 3730 sequencers. The trick to it seems to be to use the recipe given in step IV (above) for the 1.5 *M* NaOAc 250-m*M* EDTA solution.

This protocol is optimized for 0.2-ml strip tubes and a titer plate centrifuge but can be used with 96-well plates with only minor modifications.

1. If evaporation has occurred in any of the tubes, add dH_2O until it matches the others. Total volume should be about 10 μl.
2. Add 1 μl of 1.5 *M* NaOAc, 250 m*M* of EDTA (pH 8.0, from step IV), using the 0.5–10.0 μl multichannel pipetter and mix by pipetting up and down (i.e., sklooshing). (*Note:* 1.5 *M* NaOAc pH should *not* be adjusted to pH 5.2).
3. Add 40 μl of 95% ethanol using the 5–50 μl multichannel pipetter (dripping down the sides of the tubes, tips do not need to be changed between samples).
4. Recap the tubes, invert several times, and incubate for 15 min at −20°.
5. Centrifuge at 1500*g* for 45 min.
6. Remove caps, setting them aside on a clean Kim wipe.
7. Ensure the 96 deep-well block (*note:* Use the 96 deep-well blocks with *square* holes; these came from a Qiagen DNA prep kit) is dry by whipping out any liquids.
8. Carefully place a dry 96 deep-well block (*note:* Use the 96 deep-well blocks with *square* holes; these came from a Qiagen DNA prep kit) over the top of the tubes and flip (i.e., invert), leaving the tube holder in place over the tubes.
9. Centrifuge at 300*g* for 1 min, balancing with an empty deep-well block and tube holder.

10. Carefully pull the tubes straight up, off the 96 deep-well block. If any of the tubes ''stick,'' put them back into the holder in the correct orientation.

11. Recap the tubes and store them at -20° (*non*–frost-free freezer) until ready to sequence.

12. The reactions are stable for many weeks at this point. It is best to store them frozen and away from light.

Concluding Remarks

At this point, the DNA can be sequenced on several commercially available DNA sequencers (e.g., ABI, Amersham Biosciences, or Spectrumedix). Following DNA sequencing, vector and linker sequences should be removed. We screen sequences for microsatellite repeats using a simple program, Ephemeris 1.0, written in Perl by N. Dean Pentcheff (download from http://www.uga.edu/srel/DNA_Lab/programs.htm). Sequences containing microsatellites identified on at least one strand are processed further. Both strands are then contiged and edited to ensure accuracy of the sequence. After editing, primers for PCR are designed from the sequences flanking DNA using standard methodology or a three-primer system (Boutin-Ganache *et al.*, 2001); see *5′PrimerTags3.doc* at http://www.uga.edu/srel/DNA_Lab/protocols.htm for details).

All protocols used in our lab are available by following links from http://www.uga.edu/srel/ or http://gator.biol.sc.edu/. Updates to this protocol will be posted on the SREL DNA lab web site (http://www.uga.edu/srel/DNA_Lab/protocols.htm). Additional background information, steps in obtaining genotypes from microsatellite loci, and data analysis are available in *MsatMan2000.rtf* (download from http://www.uga.edu/srel/DNA_Lab/protocols.htm) and the microsatellite list-serve and associated web pages (http://www.uga.edu/srel/Microsat/Microsat-L.htm).

Acknowledgments

We have been working on this refinement for several years. Too many people and funding agencies have contributed to the protocol presented here to acknowledge everyone. We would, however, especially like to thank and acknowledge Ryan Thum, Kari Schilling, Pam Svete, Susanne Hauswaldt, Lisa Davis, Cris Hagen, and the many visiting students to our labs at SREL and USC, as well as the students enrolled in Biology 656 at USC for their contributions. Financial assistance was primarily provided by award DE-FC09-96SR18546 from the Environmental Remediation Sciences Division of the Office of Biological and Environmental Research, U.S. Department of Energy to the University of Georgia Research Foundation, with additional support from projects funded by DOE, NIH, NOAA, NSF, USDA, and various state agencies.

References

Armour, J. A. L., Neumann, R., Gobert, S., and Jefferys, A. J. (1994). Isolation of human simple repeat loci by hybridization selection. *Human Mol. Gen.* **3,** 599–605.

Boutin-Ganache, I., Raposo, M., Raymond, M., and Deschepper, C. F. (2001). M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele sizing methods. *BioTechniques* **31,** 24–28.

Fisher, D., and Bachmann, K. (1998). Microsatellite enrichment in organisms with large genomes (*Allium cepa L.*). *BioTechniques* **24,** 796–802.

Goldstein, D. B., and Schlotterer, C. (1999). ''Microsatellites: Evolution and Applications.'' Oxford Press, Oxford, UK.

Hamilton, M. B., Pincus, E. L., DiFiore, A., and Fleischer, R. C. (1999). Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques* **27,** 500–507.

Hauswaldt, J. S., and Glenn, T. C. (2003). Microsatellite DNA loci from the diamondback terrapin (*Malaclemys terrapin*). *Mol. Ecol. Notes* **3,** 174–176.

Holton, T. A., and Graham, M. W. (1991). A simple and efficient method for direct cloning of PCR product using ddT-tailed vectors. *Nucleic Acids Res.* **19,** 1156.

Kandpal, R. P., Kandpal, G., and Weissman, S. M. (1994). Construction of libraries enriched for sequence repeats and jumping clones, and hybridization selection for region-specific markers. *Proc. Natl. Acad. Sci. USA* **91,** 88–92.

Kijas, J. M. H., Fowler, J. C. S., Garbett, C. A., and Thomas, M. R. (1994). Enrichment of microsatellites from the citrus genome using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles. *BioTechniques* **16,** 656–662.

Korfanta, N. M., Schable, N. A., and Glenn, T. C. (2002). Isolation and characterization of microsatellite DNA primers in burrowing owl (*Athene cunicularia*). *Mol. Ecol. Notes* **2,** 584–585.

Marchuk, D., Drumm, M., Saulino, A., and Collins, F. S. (1991). Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Res.* **19,** 1154.

Moore, S. S., Sargeant, L. L., King, T. J., Mattick, J. S., Georges, M., and Hetzel, J. S. (1991). The conservation of dinucleotide microsatellites among mammalian genomes allows the use of heterologous PCR primer pairs in closely related species. *Genomics* **10,** 654–660.

Ostrander, E. A., Jong, P. M., Rine, J., and Duyk, G. (1992). Construction of small-insert genomic DNA libraries highly enriched for microsatellite repeat sequences. *Proc. Natl. Acad. Sci. USA* **89,** 3419–3423.

Pepin, L., Amigues, Y., Lepingle, A., Berthier, J., Bensaid, A., and Vaiman, D. (1995). Sequence conservation of microsatellites between Bos Taurus (cattle), Capra hircus (goat) and related species. Examples of use in parentage testing and phylogeny analysis. *Heredity* **74,** 53–61.

Primmer, C. R., Moller, A. P., and Ellengren, H. (1996). A wide-range survey of cross species microsatellite amplifications in birds. *Mol. Ecol.* **5,** 365–378.

Prince, K. L., Glenn, T. C., and Dewey, M. J. (2002). Cross-species amplification among peromyscines of new microsatellite DNA loci from the oldfield mouse (*Peromyscus polionotus subgriseus*). *Mol. Ecol. Notes* **2,** 133–136.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). ''Molecular Cloning: A Laboratory Manual.'' Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sambrook, J., and Russell, D. W. (2001)). ''Molecular Cloning: A Laboratory Manual.'' Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schable, N. A., Fischer, R. U., and Glenn, T. C. (2002). Tetranucleotide microsatellite DNA loci from the dollar sunfish (*Lepomis marginatus*). *Mol. Ecol. Notes* **2,** 509–511.

Tautz, D. (1989). Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res.* **17,** 6463–6471.

Weber, J. L., and May, P. E. (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am. J. Human Genet.* **44,** 388–396.

Weissenbach, J., Gyapay, G., Dib, C., Vignal, A., Morissette, J., Millasseau, P., Vaysseix, G., and Lathrop, M. (1992). A second-generation linkage map of the human genome. *Nature* **359,** 794–801.

Zane, L., Bargelloni, L., and Patarnello, T. (2002). Strategies for microsatellite isolation: A review. *Mol. Ecol.* **11,** 1–16.

Zhu, Y., Queller, D. C., and Strassmann, J. E. (2000). A phylogenetic perspective on sequence evolution in microsatellite loci. *J. Mol. Evol.* **50,** 324–338.

[14] Use of Microsatellites for Parentage and Kinship Analyses in Animals

By MICHAEL S. WEBSTER and LETITIA REICHART

Abstract

Microsatellite markers are quickly becoming the molecular marker of choice for studies of parentage and kinship in animals. In this chapter, we review methods and give protocols for screening potential microsatellite markers, as well as protocols for genotyping individuals with useful markers once they have been identified. In addition, we explain how microsatellites can be used to assess parentage and kinship, give basic analytical methods, and briefly review more sophisticated approaches that can be used to circumvent many of the problems that arise in any real empirical study.

Introduction

The application of molecular genetic methods to the study of natural populations has allowed researchers to directly examine kinship and parent–offspring relationships and thereby ushered in a revolution in our understanding of mating systems and social behavior. During the early phase of this ''molecular revolution,'' most researchers used protein allozymes or multilocus DNA fingerprinting (Burke, 1989). Microsatellites have become the marker of choice for studies of kinship and parentage.

0076-6879/05 $35.00