

Isolating Microsatellite DNA Loci*

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Glenn & Schable_update4. Last updated in 2007.

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 labs as well as 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. These protocols have been successfully used in our lab to isolate microsatellite DNA loci from more than 125 species representing all eukaryotic kingdoms. Using these protocols, the total time to identify candidate loci for primer development from most eukaryotic species can be accomplished in as little as one week.

*This information is based on (i.e., PLEASE CITE [using either style]):

Glenn, T.C. and N.A. Schable. 2005. Isolating microsatellite DNA loci. *Methods in Enzymology* 395:202-222.

or

Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. Pp. 202-222 In: *Methods in Enzymology 395, Molecular Evolution: Producing the Biochemical Data, Part B.* (eds Zimmer EA, Roalson EH). Academic Press, San Diego, CA.

Microsatellite DNA loci have become important sources of genetic information for a variety of purposes (Goldstein and Schlotterer, 1999; Webster and Reichart 2004). To amplify microsatellite loci by 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 yet been isolated from many additional species of interest and remain to be developed.

Many different 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 (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 prior to cloning, and therefore 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) among others (see Zane *et al.*, 2002 for a review). 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, 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 Hamilton, et al.’s (1999) SNX linker 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 following 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.

I. Extracting DNA

Goal: to isolate ~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, however, 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 home-made 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 below that. Thus, you may need to do an ethanol precipitation of the DNA recovered from a Qiagen kit (or comparable kits and/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 μ L of DNA with 3 μ L of TLE (10 mM Tris pH 8, 0.2 mM EDTA), 2 μ L of loading buffer (see Sambrook *et al.*, 1989 or Sambrook and Russell 2001), 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 <10 DNA samples, and the best 2 among those are used. A small to modest amount of DNA smearing down below 5,000 base pairs (bp) is generally fine. If a substantial proportion of the DNA is less than 5,000 bp (especially if <2,000 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 know which individual any particular clone (locus) came from. 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 2 species - ideally the two least related (i.e., most distant phylogenetically).

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, yet still retain 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 BstU I. These restriction enzymes can be purchased through New England BioLabs, Inc (NEB). To learn more about these or other enzymes, NEB has an informative website at <http://www.neb.com>. Any frequent cutting restriction enzyme that leaves a blunt end could be used, though it is best if they are heat labile and work in NEB buffer #2.

It is best to begin by setting up a digest on 2 DNA samples using Rsa I. If the resulting smear is not continuous with most of the DNA ranging from approximately 300 bp to 1000 bp, then it is wise to attempt another digest on uncut DNA using BstU I.

Recipe:

Rsa I or BstU I *

2.50µL NEB 10x Ligase Buffer (note: heat briefly to 50°C to get all components in solution)

0.25µL 100x BSA (Bovine Serum Albumin supplied with enzymes from NEB)

0.25µL 5M NaCl (50 mM final)

1.00µL Rsa I (NEB catalog # R0167S) **or** BstU I (NEB catalog # R0518S)

1.00µL Xmn I (NEB catalog # R0194S; note: Xmn I can be added at step III.2 instead)

20.0 µL** genomic DNA (100 ng/µL)

*Rsa I recognizes GT[^]AC and BstU I recognizes CG[^]CG; so one may work better than another in any particular organism. Hae III (GG[^]CC, catalog # R0108S) 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 are: EcoR 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).

****Assumes a DNA concentration of ~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 prior to enrichment (especially if the total amount of DNA available is less than 100 ng). It is important to note that amplifying the linker ligated DNA prior to enrichment may bias the enrichment results.**

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 if 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 BstU I, multiply the volume of each of the components above the dotted line by the number of DNA samples to be digested plus 1/2 a sample to account for pipetting error and add to a 1.5ml tube.
2. Prepare the restriction enzyme digest for Rsa I and BstU I by adding 5μL of master mix into a new tube (0.2 mL or 0.5 mL depending on thermal cycler available) and use a thermal cycler for all incubations. Add 20μL of DNA to each tube. Pipette up and down to mix the solution.
3. Incubate all samples (Rsa I and BstU I) at 37°C for 30 – 60 min.

4. While the restriction digest is incubating, pour a 1% agarose gel, including Ethidium Bromide (see Sambrook et al., 1989). Also, while the restriction digest is incubating, make the double-stranded linkers (III.1 below).
5. Set aside a small aliquot (4 μ L) 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.3 below.

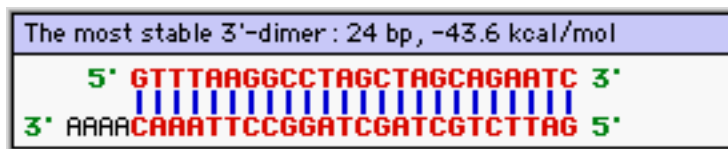
III. Ligating Linkers to DNA Fragments

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 "pig-tail" to facilitate non-template 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 2 enrichments per linker-ligated DNA. There is enough linker ligation for 3 enrichments using the recipes below. If one plans to perform more than 3 enrichments, then 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'pGATTCTGCTAGCTAGGCCTTAAACAAAA



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 picture.

Detailed Steps:

1. Preparation of double stranded (ds) SuperSNX linkers: [note: This step can be done while the restriction digest is incubating (step II.3) or earlier; batches of linker can be made and frozen for future use.]

Mix equal volumes of equal molar amounts of SuperSNX24 and SuperSNX24+4p primers (e.g., 100 μ L of 10 μ M each). Add salt to a final concentration of 100 mM (i.e., 4 μ L of 5M

NaCl for 200 μL of primers). Heat this mixture to 95°C , 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 10x 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, then make a master mix of the components above the dashed line and **add them (10 μL of mix) to the cut DNAs.**

3. Incubate at room temperature for 2⁺ hrs. or ideally at 16°C overnight.
4. While the ligation is proceeding, run the small aliquots of restriction enzyme digested DNA (from step II.5 above) on the 1% minigel (from step II.4 above) to ensure the DNA samples were successfully digested. A successful reaction should yield a smear of fragments centered at approximately 500bp, but at least with most fragments at or below 1,000 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 10x PCR buffer (optimal buffer for *Taq* used below)

2.5 μL BSA (bovine serum albumin, 250 $\mu\text{g}/\text{mL}$ -> 25 $\mu\text{g}/\text{mL}$ final)

1.3 μL SuperSNX-24 (10 μM -> 0.5 μM final)

1.5 μL dNTP's (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 μ L Linker ligated DNA fragments

It is correct that only 1 primer is used (see section V note 1).

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

Cycling: 95°C for 2 min.; then, 20 cycles of 95°C for 20 sec., 60°C for 20 sec., 72°C for 1.5 min. Hold at 15°C. Note: the same program as the enrichment recovery (step V below) may be used, but it takes longer.

6. Run 4 μ L of PCR product on a 1.0% minigel to see if the linker-ligation was successful, using a 100bp ladder as a size standard. A successful reaction should yield a smear of fragments centered at approximately 500bp. 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°C 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 μ g of 1 kb fragments = 3.3 pmol fragment ends. Also, 1 μ L of X μ M linkers = X pmol of linker ends.
- The *Xmn I* prevents the dimerization (self ligation) of linkers, thus it is vital for success.

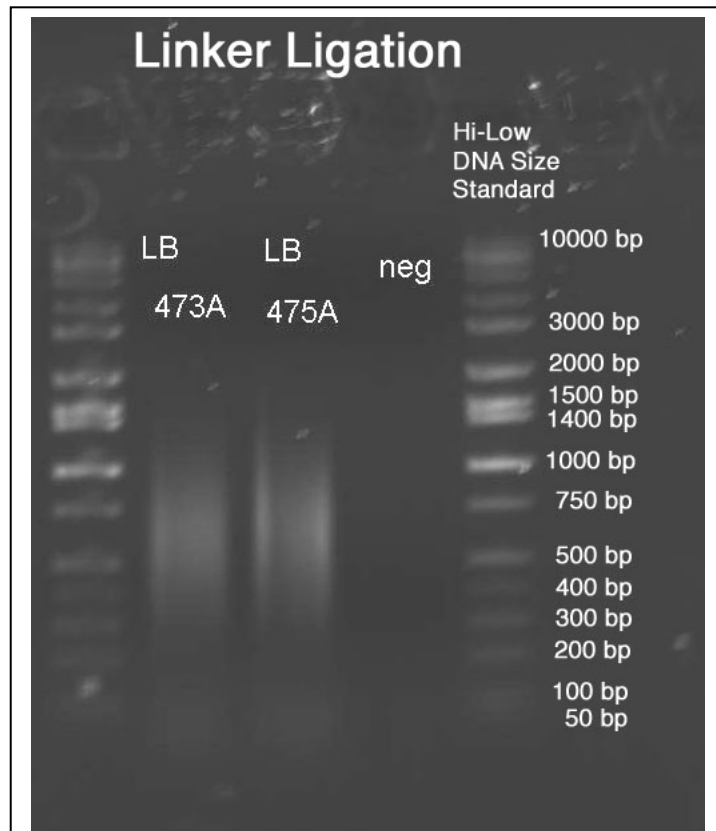


Figure 1. Image of successful Linker Ligations. Note the continuous smear from ~300 to ≥ 1000 bp. The same size standard will be used in subsequent figures.

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 only a need for 2 enrichments per linker-ligated DNA (see note III 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 pH8, 2 mM EDTA] and twice in 1x Hyb Solution) – note: each 50 μ L of Streptavidin M-280 Dynabeads (Invitrogen [formerly Dynal]; catalog No. 112-05D) 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. NOTE (March 2008): We are testing Dynabeads MyOne Streptavidin C1Beads because these have much lower non-specific binding than the M-280 beads. Another approach to reduce non-specific binding is to wash the M-280 beads with 1% I-Block™ Protein-Based Blocking Reagent (catalog T2015, ~\$40) from Applied Biosystems (.).

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

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

Washing Solutions: 2xSSC, 0.1%SDS (warmed to get everything into solution)
1xSSC, 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). The mixture is made by taking individual oligos all at the same concentration, mixing equal volumes, and diluting from there [e.g., for 5 oligos, each at 100 μ M; mix 100 μ L of each (=500 μ L at 100 μ M total conc., 20 μ M each); then dilute 1:99 with TLE (10 mM Tris pH 8, 0.2 mM EDTA) to get a final total concentration of 1 μ M]. If the oligos start at different concentrations, then adjust the volumes to get equal concentrations in the mix. 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_M 's). We use oligos purified by standard desalting methods (i.e., no additional purification by 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, thus it is critical to ensure the amount of biotin (estimated from the oligo concentration) added is FAR below the bead binding capacity. We generally use the following 3 biotinylated oligo for enrichments.

Mix 2 = (AG)₁₂, (TG)₁₂, (AAC)₆, (AAG)₈, (AAT)₁₂, (ACT)₁₂, (ATC)₈;

Mix 3 - (AAAC)₆, (AAAG)₆, (AATC)₆, (AATG)₆, (ACAG)₆, (ACCT)₆, (ACTC)₆, (ACTG)₆;

Mix 4 - (AAAT)₈, (AACT)₈, (AAGT)₈, (ACAT)₈, (AGAT)₈.

NaOAc EDTA Solution: To a 50 mL conical, make 20 mL of 3M NaOAc from the dry chemical stock. Do not adjust the pH! Add 20 mL of 500 mM EDTA, pH 8. This makes a solution that is 1.5 M NaOAc and 250 mM 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

25.0μL 2x Hyb Solution (warmed to get everything into solution)

10.0 μL Biotinylated microsatellite probe (mix of oligos at 1 μM total; see note above)

10.0μL Linker ligated DNA from step III (or PCR product if <2 μg DNA initially used)

5.0μL dH₂O

50.0μL Total

2. Use thermal cycler program **OLIGOHYB**. This program denatures the DNA + probe mixture at 95°C for 5 minutes. It then quickly ramps to 70°C and steps down 0.2°C every 5 seconds for 99 cycles (i.e., 70°C for 5 sec., 68.8°C for 5 sec., 68.6°C for 5 sec., ... down to 50.2°C),

and stays at 50°C for 10 minutes. It then ramps down 0.5°C every 5 seconds for 20 cycles (i.e., 50°C for 5 sec., 49.5°C for 5 sec., 49°C for 5 sec.,...down to 40°C), and finally quickly ramps down to 15°C. 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 50µL of Dynabeads (Invitrogen). Resuspend the beads in their original tube, transfer to a 1.5 mL tube. Add 250 µL of TE. Shake. Capture beads using the Magnetic Particle Concentrator (MPC) (Invitrogen [formerly Dynal]; catalog No. 120-20D). Repeat with TE, and twice with 1xHyb Solution. Resuspend the final beads in 150 µL of 1xHyb Solution.
4. Pulse-spin your DNA+probe mix and add all of it to the 150µL of washed, resuspended Dynabeads (i.e., to the 1.5mL tube).
5. Incubate on rotator, or sideways in orbital shaker on slow speed at room temp. for 30+ 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 2xSSC, 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 trouble-shooting purposes). Resuspend beads well (i.e., flick or gently vortex) in next wash each time.
8. Wash two additional times using 400µL 1x SSC, 0.1% SDS.
9. Wash two final times using 400µL 1xSSC, 0.1% SDS and heating the solution to within 5-10°C of the T_m for the oligo mix used (usually 45 or 50°C). *Note: More stringent washes may increase the relative proportion of long microsatellite sequences in cloned colonies, however, they may also cause the loss of microsatellite sequences via undesired elution during washing.
10. Add 200µL TLE (10 mM Tris pH 8, 0.2 mM EDTA), vortex and incubate at 95°C for 5 minutes. 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°C 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 bead 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 store in the -20°C 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 left.
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 100% sure that the DNA is in solution. Inadequate rehydration is the most common reason for failure of the next step.

V. PCR Recovery of Enriched DNA

Goal: to increase the amount of "pure Gold" DNA. If one plans to do serial (double) enrichments, then the PCR products generated from this step will be used for the second enrichment. When doing serial enrichments, it is wise to set up replicate PCR's and combine them prior to the next enrichment.

Detailed Steps:

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

- 2.5 μ L 10x PCR buffer (optimal buffer for *Taq* used below)
- 2.5 μ L BSA (bovine serum albumin, 250 μ g/mL -> 25 μ g/mL final)
- 1.5 μ L dNTP's (2.5 mM each -> 150 μ M final)
- 1.3 μ L SuperSNX-24 (10 μ M -> 0.5 μ M final)
- 2.0 μ L MgCl₂ (25 mM -> 2.0 mM final)
- 13.0 μ L dH₂O
- 0.2 μ L *Taq* DNA Polymerase (5 units/ μ L)

2.0 μ L Eluted DNA fragments ("pure Gold") [note: ensure gold pellet has hydrated for 20⁺ min. – 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 dashed line and add them (23 μ L of mix) to the Eluted DNAs (2 μ L). It is often wise to perform a second PCR, using half as much Eluted DNA (i.e., 1.0 μ L of eluted DNA + 1.0 μ L of dH₂O).

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

Note: it is correct that only 1 primer is used (the SuperSNX-24 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, then it is easier to visualize why using only 1 primer works.

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5 ' ForwardPrimer :: DNAofInterest :: ReversePrimer3 '  
3 ' ReversePrimer :: DNAofInterest :: ForwardPrimer5 '
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2. Run 4 μ L of PCR product on a 1.0% minigel next to a 100bp ladder as a standard to verify if DNA recovery was successful (the smear of fragments should be visible, centered at approximately 500bp). The rest of the PCR product can be used for cloning in the next step, or used in place of the linker-ligated DNA as starting material for serial (i.e., double) enrichment (step IV.1.).

Note: If bands are visible, but there is also a clear background smear, then it is often OK to proceed. The bands often times represent repetitive elements. All sequences should be searched against each other to find identical clones. If bands are visible, but there is no or very little background smear then it is possible that few msat 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 PCR reactions yields only a few bands, then enrich using a different mix of oligos.

Optional – for Trouble Shooting and to verify enrichment success: Use 2 μ L of PCR product for a dot blot (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 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., 5x, 10x, 50x) 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 at all, and the

Linker-ligated DNA is very dark, then it may be wise to use a different mix of microsatellite oligos and/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).

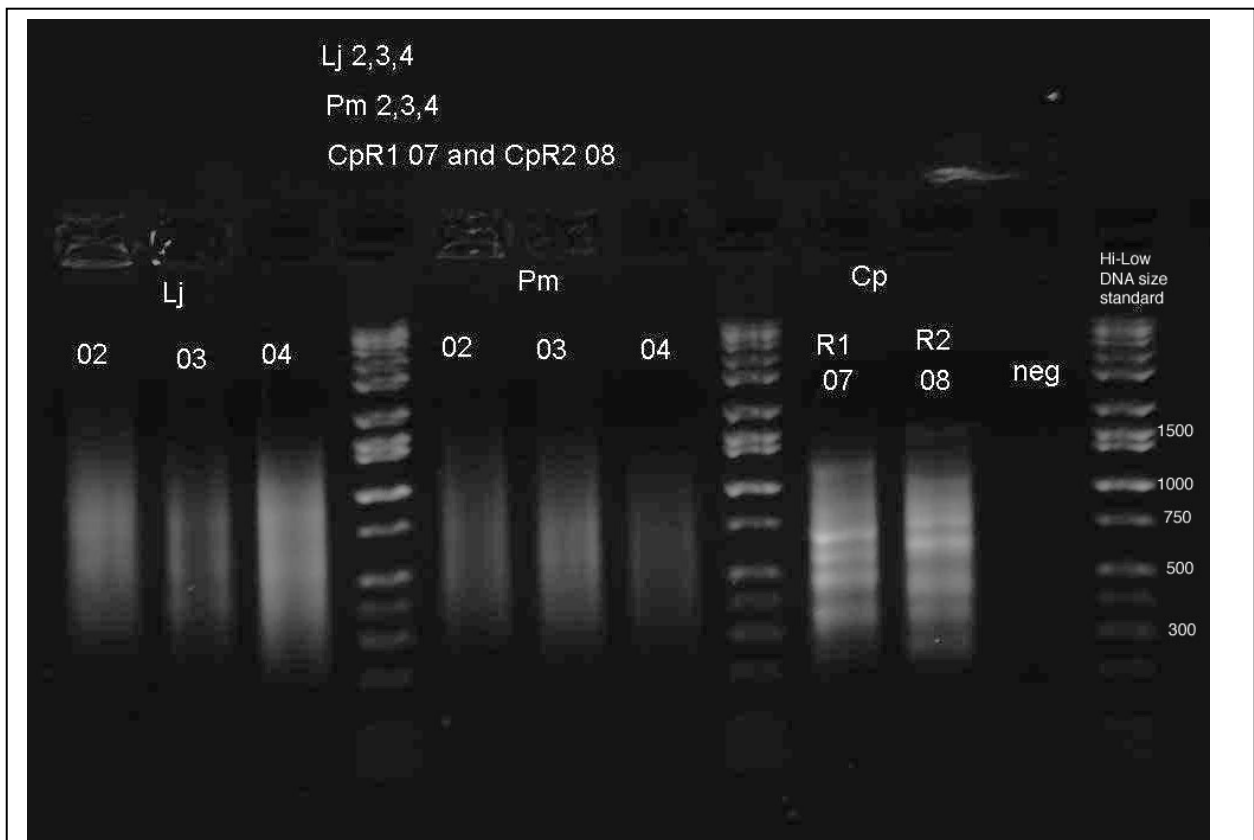


Figure 2. Gel Image of Successful Enrichments. Three different experiments with 3 different organisms (Lj, Pm, and Cp) are shown. The Lj and Pm enrichments are for standard enrichments using 3 different mixes of biotinylated oligos (see notes above). The Cp enrichments are from a limited pool of starting DNA – note the “bands” that appear in these 2 – the bands are indicative of a few repetitive elements dominating the enrichment.

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 (e.g., 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, then one can purchase the 20 reaction kit (K4500-01), rather than the 40 reaction kit.

We will commonly combine recovery PCR products prior to ligation (i.e., add equal amounts of PCR product into one tube & then take out the appropriate amount from there). This minimizes the number of independent libraries that must be constructed. Any of the products from the species Lj and Pm in Figure 2 could be combined. It is best to avoid any enrichments that look like those of species Cp in Figure 2 (i.e., don't use them for ligations).

Detailed steps:

1. Follow the directions supplied with Invitrogen's Topo-TA cloning kit exactly! If another TA cloning protocol is used, then follow the appropriate directions for ligation and transformation.
2. Warm S-GalTM/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, then 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

following 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).

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 are 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 the TA cloning kits from Invitrogen are used. Other standard transformation protocols (Sambrook *et al.*, 1989; Sambrook and Russell, 2001).

Detailed Steps:

1. Follow Invitrogen’s Topo-TA cloning kit exactly!
2. Following the 1 hour incubation in SOC, plate out 25µL of Transformed bacteria onto 2 plates and 50 µL of Transformed bacteria onto 2 other plates. This will ensure 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 go ahead and plate out the entire amount, or one may save the remaining transformed bacteria in broth (at 4°C) 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 µL will, however, be reduced by plating at a later time).
3. Grow colonies overnight at 37°C.

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 μ L of ampicillin (50mg/mL stock) to a 50 mL conical tube full of broth. Add 300 μ L 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° C with semi-vigorous shaking. It is often beneficial to incubate an additional 24 hours (~40 hours total) to achieve high density cell growth.

Day 2

5. Set up the following 25 μ L PCR reactions:

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

275.00 μL 250 $\mu\text{g}/\text{mL}$ BSA (Bovine Serum Albumin)
275.00 μL 10X PCR reaction 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 dNTP's (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 (i.e., bacteria grown up in LB broth; step 4 above)

If setting up fewer than 96 reactions, then the 25 μL reaction recipe (per reaction) is:

2.50 μL 250 $\mu\text{g}/\text{mL}$ BSA (Bovine Serum Albumin)
2.50 μL 10X PCR reaction 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 2.5mM dNTP's (2.5 mM each)
12.80 μL dH_2O
0.20 μL Taq DNA Polymerase (2.5 Units/ μL)
1.50 μL DNA template (i.e., bacteria grown up in LB broth; step 4 above)

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

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

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 μ L of the M13/bacterial PCR product on the agarose gel along with a 100bp ladder and 2 μ L of several different Lambda concentration standards (λ 10 ng/ μ L, λ 25 ng/ μ L, λ 50 ng/ μ L and λ 100 ng/ μ L). A 10 μ L multi-channel 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 Volts for approximately 30-40 minutes.
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/over-integrated (red). If DNA concentration varies a lot (i.e., the brightest samples are red when you're exposing enough to see the dimmest samples, then save multiple exposures).
10. The desired insert range is from 300 – 1000 bp. Because the pCR2.1 vector contains ~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 PCR reactions have been examined:

11. To a 50 mL conical tube, add 15 mL of glycerol, fill to 50 mL with LB broth, and add 50 μ L of ampicillin (50mg/mL stock). Mix thoroughly by shaking.
12. Remove the bacterial cultures from the refrigerator or incubator.

- Using the multichannel pipette, add 300 μ L 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° C.

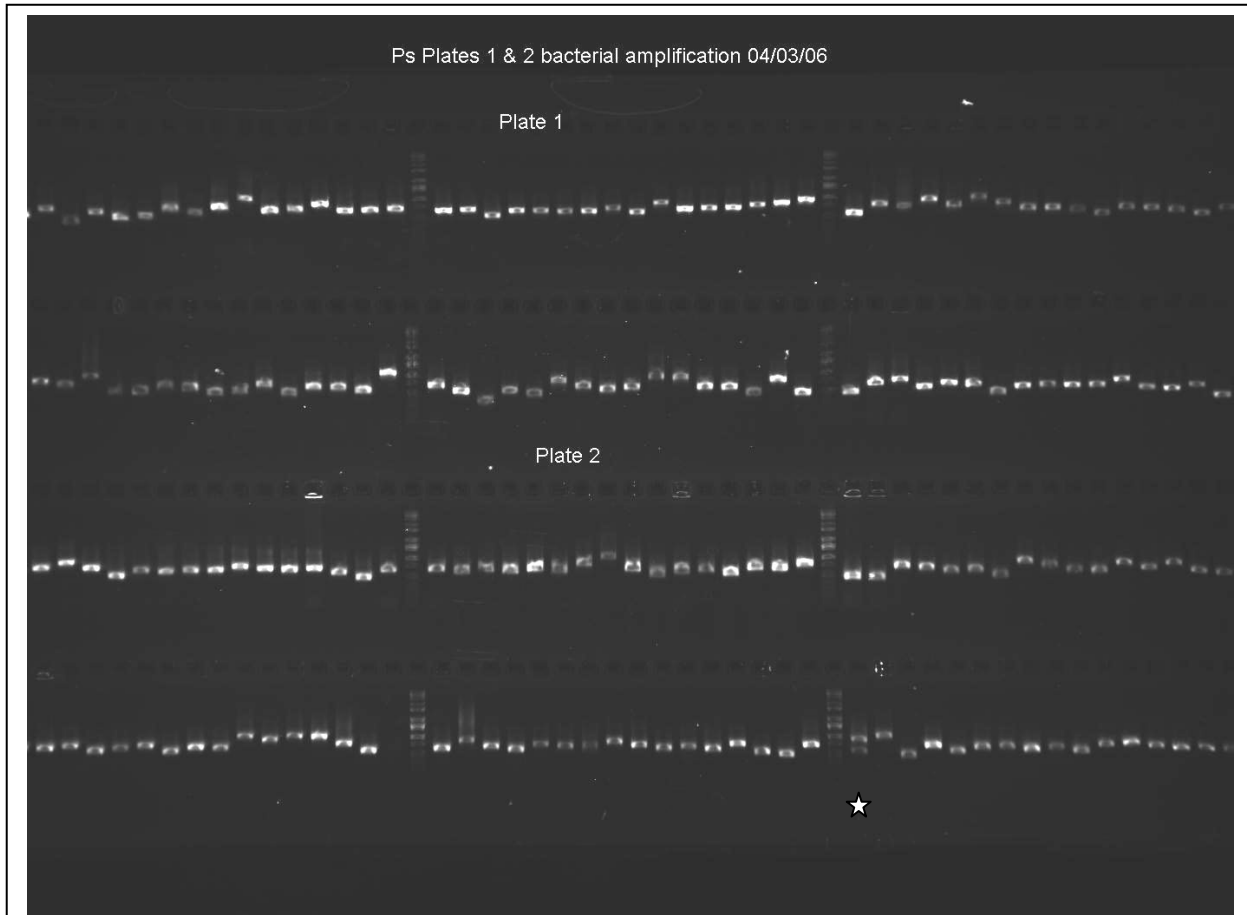


Figure 3. Typical PCR results for two 96-well plates of clones. Each row has 48 PCR products and 2 size standards. Note that the products vary in size. If you look very closely (especially at the middle section of row 3 [3rd from the top]), many bands appear to be doublets – this is normal. If you examine row 4 (bottom row) the first sample to the right of the size standard on the right (there is star below this sample) – you will see 2 well differentiated bands – this is probably from picking 2 clones into the same well – sequencing will fail for this sample, as well as the other sample that did not amplify (also row 4).

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, then dilution (i.e., using no more than 0.5 μL of PCR product) is efficient and usually works well. However, cleaning the PCR reaction using Exonuclease I and Shrimp Alkaline Phosphatase (SAP or its variants) is a preferred option, which improves consistency among experiments and researchers in our lab.

Materials and Solutions:

Home-made ExoAP: Combine equal volumes of Exonuclease I (NEB Catalog #M0293L; 20 units/ μL) with Antarctic Phosphatase (NEB Catalog #M0289L; 5 units/ μL]. Keep this in the -20 freezer as a stock solution.

1. Quantify PCR product concentration and size. For a single sequencing reaction, the desired amount of template is 10ng 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 at least 2 sequencing reactions. Use the imaging results saved from step VIII.12 above (see Figure 3).

2. When ready to purify reactions for a plate, combine:

22 μL	10X AP buffer (10x PCR buffer may be used instead)
44 μL	ExoAP (from above)
374 μL	dH ₂ O

54 μL of this mix into each tube of an 8-tube strip

4.0 μL	Mix from the strip tubes in each well of a 96-well plate
4.0 μL	PCR product from colony PCR (spin plate to remove condensation from the lid.)

(Note: volumes can be adjusted to account for differences in amplification & lengths of the PCR products, so that the molar concentrations are approximately equal, however, in practice we find this recipe generally works fine without such adjustment).

3. Incubate the samples at 37°C for 15 minutes, 80°C for 15 minutes then hold at 15°C. The samples are now purified, and ready for use as sequencing reaction template.

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 plates, but also works fine 0.2 mL strip tubes. The recipe given is for 1/16th reactions (half the normal volume & using 1/8th of the BigDye recommended for “standard” sequencing). We find that increasing the amount of BigDye beyond this does not generally improve results (% of successful reactions or read length). Reactions above 1/8th of are generally too strong for our 3130 (and thus also 3730 sequencers).

1/16th Reaction Recipe:

3.5µL **2.5x** Sequencing Dilution Buffer*

0.5µL BigDye Terminator v 3.1 mix**

1.0µL Primer*** (3.3 µM)

---(make a master mix; if the template concentration is constant, then add the water here)---

2.0µL DNA template (**10 ng per 100bp of product length; adjust volume as appropriate**)

3.0µL H₂O *** (**adjust volume as appropriate to make total of 10.0 µL**)

1/16th Recipe for a 96-well plate:

385 µL **2.5X** buffer

330 µL dH₂O

55 uL BigDye Terminator v 3.1 mix

110 µL Primer*** (3.3 µM)

108 µL of this mix into each tube of an 8-tube strip

8.0 µL Mix from the strip tubes into each well of a 96-well plate

2.0 µL ExoAP PCR product from colony PCR (spin plate prior to removing the lid)

Notes:

*BigDye Dilution Buffer is shipped at 5x, mix 1:1 with water for 2.5x. A Home-made version of

5x Sequencing Dilution Buffer is - 400mM Tris-HCl, pH 9.0; 10mM MgCl₂

**Even more dilute reactions may be used. Adjust BigDye & Dilution buffer appropriately.

***M13 Forward or Reverse, or other primers closer to the insertion site, as appropriate.

****If your templates are consistent in size and concentration, add the water to your master mix so that you add a constant volume of template.

Thermal Cycling: ≥ 39 cycles of 96°C for 10 sec., 50°C for 5 sec., 60°C for 4 min. Hold at 15°C. Note: no initial denaturation is necessary

Place the tubes in a 96-well holder and store the reactions at -20°C 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.

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 up your sequencing reactions. Column purification (Sephadex G50/Centri-Sep columns from Princeton Separations [see additional protocols]) 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 v3.1 manual includes a protocol very similar to one below, and one where no NaOAc is used. We used the no NaOAc protocol at UGA for sequences going on the ABI 3700 with very good success. We use the following protocol at SREL with good success. The trick to it seems to be when making up the 1.5M NaOAc + 250mM EDTA (pH 8), you can use your normal stock of 0.5 M EDTA (pH8), but you can **NOT** use a stock of 3 M NaOAc at pH 5.2. Simply make up 25 mL of 3 M NaOAc (from the powder) in a 50 mL conical tube. To that solution, add 25 mL 0.5 M EDTA, then make small aliquots & keep them in the freezer.

Plate Protocol:

1. If evaporation has occurred in any of the tubes, add ddH₂O until it matches the others. Total volume should be $\sim 10\mu\text{L}$.
2. Add 1 μL of 1.5M NaOAc + 250mM EDTA (pH 8), using the 0.5-10 μL multi-channel pipetter and **mix by pipetting (i.e., sklooshing)**.
3. Add 40 μL of 95% ethanol using the 5-50 μL multi-channel pipetter (by dripping down the sides of the tubes, you don't need to change tips between samples).

4. Place mat on plate, place on Microplate Genie for 1+ min., and then let sit for 15 min. at –20°C.
5. Centrifuge at 1,500xG for 45 min.
6. Turn plate upside down over the sink to dump out liquid.
7. Place plate upside down on a paper towel and centrifuge at 300xG for 1 minute to remove remaining liquid.
8. Cover the plate with the mat, ensure it is properly labeled, wrap in aluminum foil and store at –20°C (**non**-frostfree freezer) until ready to sequence.

The reactions are stable for many weeks at this point. The reactions can be safely mailed overnight at ambient temperature to a sequencing facility. It is, however, best to keep them at -20C (in a **non**-frost-free freezer) and away from light to the extent possible, especially if storing for days, weeks, or months. We have stored samples at -20C for up to 2 months with no discernible difference in signal (i.e., the results looked normal).

Notes on Precipitation: NaOAc is a salt (counter ion) to bring the DNA out of solution during precipitation. EDTA binds the dNTPs and keeps them from precipitating (which is the goal—we want to get rid of the dNTPs). Using the original ABI recipe (no NaOAc or EDTA) depends on having precise final concentrations of EtOH to precipitate the DNA but not the dNTPs. Adding NaOAc makes the final concentration of EtOH less critical but helps ALL DNA (including dNTPs) precipitate. So you need EDTA when using NaOAc to keep the dNTPs in solution.

Conclusion

At this point, the DNA can be sequenced on several commercially available DNA sequencers (from e.g., Applied Biosystems, Amersham Biosciences, or Spectrumedix). Following DNA sequencing, vector and linker sequences should be removed. Below is the linker (Super SNX) and vector (pCR2.1 Topo from Invitrogen) sequence information. The • shows where the insert sequence from your critter should begin.

Super SNX

GTTTAAGGCCTAGCTAGCAGAATC•GATTCTGCTAGCTAGGCCTTAAAC

pCR2.1 TOPO

CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACT
AGTAACGGCCGCCAGTGTGCTGGAATTCGGCTT•AAGCCGAATTCTGCAGATATCCA
TCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCCTATAGTGAG
TCGTATTACAATTCAGTGGCCGTCGTTTTAC

pCR2.1 Topo + SuperSNX

CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACT
AGTAACGGCCGCCAGTGTGCTGGAATTCGGCTTGTTTAAGGCCTAGCTAGCAGAATC
•GATTCTGCTAGCTAGGCCTTAAACAAGCCGAATTCTGCAGATATCCATCACACTGG
CGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCCTATAGTGAGTCGTATTAC
AATTCAGTGGCCGTCGTTTTAC

Scripts are now available to process chromatograms, find repeats, and design primers (Fukuoka et al. 2005).

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). Brant Faircloth at UGA (e-mail brant@uga.edu) has written a similar program using Python. Brant's new program will search for all 501 unique mono- to hexa-nucleotide repeats, as well as working with concatenated FASTA formatted files. Brant's program also outputs data into a format which can be easily imported into Excel.

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. Following

editing, primers for PCR are designed from the sequences flanking DNA using standard methodology or a 3 primer system (cf.(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>).

Acknowledgements

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 Cris Hagen, Ryan Thum, Kari Schilling, Pam Svete, Susanne Hauswaldt, Lisa Davis 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.

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