

Enhanced Microsatellite Genomic Library Construction Bench Version

Purpose: To construct and clone genomic libraries with increased proportions of inserts that contain tandem repeat arrays. Thus, a greater number of microsatellite repeat regions can be detected, sequenced and subsequently used to design species-specific flanking primers for microsatellite amplification.

From Fleischer, R. C. and S. Loew, 1995. Construction and screening of microsatellite-enriched genomic libraries. Pages 459-468 in J. Ferraris and S. Palumbi (eds.), Molecular Zoology: Advances, Strategies and Protocols. Wiley-Liss, New York. Based on Armour, J. A. L., R. Neumann, S. Gobert and A. J. Jeffreys. 1994. Isolation of human simple repeat loci by hybridization selection. *Human Molecular Genetics* 3: 599-605.

Users of this protocol who are successful in isolating microsatellites are asked to cite Fleischer, R. C. and S. Loew, 1995 and "M. Hamilton and R. Fleischer, personal communication" in any publications. We would also be grateful for any suggestions or improvements.

Disclaimer: Persons using this protocol do so at their own risk. Every effort has been made to verify the procedures described here and the technique has been used successfully in the Molecular Genetics Laboratory at the National Zoo on multiple occasions. However, the authors cannot be held liable for failure of the protocol to produce microsatellites. Users must exercise proper professional judgment when carrying out these procedures and are encouraged to make modifications that are appropriate for their starting materials and end goals. We have not exhaustively described every aspect of these molecular genetic procedures and required safety precautions. This protocol is not intended for those without background in molecular genetic laboratory procedures.

Detailed Protocol:

I. Processing insert DNA:

I A. Digest to completion at least 5 μ g of clean genomic DNA (from individual of heterogametic sex) with a 10-fold excess of restriction enzyme MboI, DpnII, Sau3AI or other isoschizomer that leaves a 5'GATC overhang. One can and should employ an enzyme that cuts genomic DNA so that a large proportion of fragments are in the 800 to 100 base pair range. If an enzyme other than Dpn II is used the SAU B linker must be changed to have the appropriate complementary overhang (see appendix). One can also use enzymes that leave blunt ends. For blunt ends the SAU B must have NO overhang and a 40 to 100 fold molar end excess of linkers must be added to genomic DNA during ligation. Blunt end ligation efficiency is very low so this method is not preferred. Use the manufacturer's conditions and buffer and digest from 4 h to overnight at 37°C in a total volume of 20-100 μ l:

- | | |
|------------------------------|-----------|
| 1. genomic DNA, 5-15 μ g | x μ l |
| 2. 10x buffer: | y μ l |

- | | | |
|----------------------------------|-----------|------------------|
| 3. 10-20 units of <i>DpnII</i> : | | <i>z</i> μ l |
| 4. RNase A (if necessary): | 1 μ l | |
| 5. sterile distilled water: | | a μ l |

Run 2 μ l of this digest on 1.5% agarose to check completeness of digestion. If digestion is not complete add more enzyme (up to 50 units).

I B. One could now **dephosphorylate** the 5' sticky ends to increase the likelihood of ligation to the linkers below by decreasing the likelihood of ligation of insert fragment sticky ends (see protocol in Sambrook et al. 1989). **This step is recommended** and will prevent self-ligation of genomic DNA (seen in III B as fragments that are larger than the original size-selected DNA). Note that use of NEB CIP at a concentration of 1/10 unit per pmole of DNA ends will be sufficient to dephosphorylate a 5' overhang (2 hour incubation). See catalog.

Add appropriate amount of CIP directly to digest (NEB CIP is active in most restriction enzyme buffers), mix and incubate at 37°C for at least 2 hours. Heat kill CIP (along with the restriction enzyme) by adding EDTA to a final concentration of 5mM and heat to 75°C for 10 minutes or 65°C for 1 hour. Phenol/chloroform extraction to remove CIP is not necessary because next step will remove any traces.

I C. Size select on agarose gel (200 to 1000 bp):

1. If necessary, precipitate digested, CIP'ed genomic DNA by adding 1/10 volume 3M NaOAc and 2 to 3 volumes of cold 100% ethanol. Spin at 4°C for 15 minutes to pellet and wash with 3 volumes of cold 70% ethanol. Redissolve dried pellet in about 10 μ l of TE (or volume that will fit in wells of a thin gel below).
2. Heat digested DNA for 10 minutes at 65°C (to melt complimentary ends) and load the entire amount on a 1.5% low melting point agarose gel in 1X TAE. Make gel as thin as possible to reduce amount of agarose (mark depth needed on comb if necessary). Include a ϕ X-*HincII* or other marker and locate the 200-1000 bp size range. Cut the band from the gel for this range and put into one or more microfuge tubes.
3. Proceed to Gelase (Epicenter Technologies), electroelution or spin through siliconized glass wool to remove agarose (using the manufacturers instructions). Resuspend DNA in about 20 μ l of TE.

II. Preparation of SAU linkers:

II A. Have complementary linkers synthesized (it saves a step if the 5' overhang linker is purchased 5' phosphorylated) so that double stranded they have a 5' overhang of CTAG [for genomic DNA digested with *Dpn II*] and can serve as **SAU linkers**:

1. SAULA: 5' -GCG GTA CCC GGG AAG CTT GG -3'
2. SAULB: 5' -GAT CCC AAG CTT CCC GGG TAC CGC-3'

e.g. 5' -GCG GTA CCC GGG AAG CTT GG -3'
 3' -CGC CAT GGG CCC TTC GAA CCC TAG-5'

Note that other enzyme/linker combinations are available. See appendix.

II B. Make a 100 μ M stock of each oligo in T.E.

Newly synthesized oligos are provided with yield information (usually the number of pmoles synthesized). Add T.E to make a 100 pmole/ μ l (also = 100 μ M) stock solution. Store oligo stocks at -20°C.

II C. Phosphorylate the 5' end of the primer with the overhang (SAULB **not** the SAULA). This step is unnecessary if the oligo was purchased with a 5' phosphate group. This assumes we are using NEB kinase; see catalog. This makes enough for 400 μ l of double stranded linker, which is a **large amount**. Scale as necessary.

1. 10X kinase buffer (provided by manufacturer)	5 μ l
2. SAULB , 1200 pmoles @ 100 pmole/ μ l	24 μ l
3. 10 mM ATP, pH 7.4 (fresh)	5 μ l
4. T4 Polynucleotide Kinase (10 U @ 10 U/ μ l) (use dilution buffer if included with PNK)	1 μ l
5. Sterile water	15 μ l
Total Volume:	50 μl

Incubate at 37°C for 2 hours. Heat to 65°C for 20 minutes to kill PNK.

II D. Combine equimolar amounts of SAULA and phosphorylated SAULB to construct a double-stranded linker which will be ligated to the digested and size-selected insert DNA from step I above.

1. Assume we will ligate 1 μ g of genomic DNA with an average size of 500 bp to the double stranded linker (see appendix). This is 6.154 pmoles of ends of genomic DNA. We will need to add about 6.154 pmoles of double stranded linker (remember that the linker has only one end per molecule).

2. A final concentration of 3 pmoles/ μ l of double stranded linker will give a good working concentration (2 μ l has about 6 pmoles). Combine 25 μ l of SAULB from step C (or 6 μ l of 100 pmole/ μ l stock if oligo was synthesized with 5' phosphate) with 6 μ l of 100 pmole/ μ l SAULA stock in a microfuge tube. Add T.E to a final volume of 200 μ l (gives 3 pmole/ μ l). Leave at room temperature for a few minutes so strands will anneal and store at -20°C.

III. Ligation of insert to linkers:

III A. Cohesive termini or "sticky end" ligation proceeds rapidly at linker to genomic fragment molar end ratios near 1:1. For blunt end ligations, however, linker to genomic fragment molar end ratios need to be in the range of 40:1 to 100:1 and a larger amount of ligase is needed. See appendix 1 for methods to calculate molar end concentrations and discussion in Sambrook et al. 1-68. Below is an **example** of a 20 μ l sticky end ligation reaction using NEB ligase buffer and enzyme. **Be sure to check manufacturer's protocol! Some buffers require addition of ATP.**

Add components in order shown below. Heat insert DNA and linkers @ 65°C for 10 mins to melt sticky ends and then place on ice.

1. sterile water	x μ l
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2. Insert DNA (1 μg)	$y \mu\text{l}$
3. double stranded linkers (1:1 to 1:2 genomic to linker molar end ratio)	$z \mu\text{l}$
4. 10 x ligation buffer (supplied w/ ATP)	2 μl
5. 1.0 unit T4 DNA ligase	1 μl

Incubate overnight @ 16°C in water bath or thermocycler. [NEB states that sticky end reactions proceed to completion within several hours. See NEB catalog.]

III B. Run 3 μl of ligation on a 1.5% agarose gel with size marker and a small amount of unligated, size selected genomic DNA. Successful ligation is confirmed by linker-ligated insert DNA in the 250-850 bp range (larger than the unligated genomic DNA). Linker dimers and tetramers (i.e., bands at about 44 and 88 bp) may be visible if ligations are blunt-ended due to the large excess of linker.

III C. The linker ligated inserts can be amplified directly from the ligation in a PCR reaction with the SAULA primer from above. **If you have a large amount of genomic DNA it is wise to limit the number of PCR cycles or skip this step entirely and proceed to enrichment. PCR will bias insert pool toward smaller sizes and more frequently occurring inserts.** Store the remainder of the insert/SAU DNA at -20°C. Here is the PCR recipe for a 50 μl reaction.

1. Sterile water:	$x \mu\text{l}$
2. Linker-ligated insert DNA:	5 μl
3. SAULA primer (10 μM stock):	5 μl
4. dNTP mix (8 mM):	5 μl
5. PCR buffer	5 μl
6. MgCl ₂ (if necessary)	$y \mu\text{l}$
7. <i>Taq</i> Polymerase (3 units):	0.3 μl

Thermal profile is: 94°C for 40 seconds; 60°C for 1 minute; and 72°C for 2 minutes; 20-40 cycles.

III D. Run a 3 μl aliquot of the amplified insert on a 1.5% agarose gel to check amplification and to quantify amounts. A bright smear between 250 and 850 bp indicates a successful amplification. Repeat amplification to get larger amounts of DNA if necessary.

IV. Construction of microsatellite repeats by ligation and amplification:

IV A. Long sequences of microsatellite repeats are constructed and used to select large microsatellite repeat arrays from among the amplified inserts. Some long microsatellite oligomers are available commercially (e.g., CA/GT from Pharmacia). Others need to be assembled via oligonucleotide ligation followed by a self-priming PCR reaction. There is a large number of di-, tri- and tetra-nucleotide repeats to choose from; it is a good idea to consult the literature to identify those that are generally found in genomes of the taxonomic group on which you work.

IV B. Oligonucleotides should be phosphorylated for ligation (alternatively oligonucleotides can be 5' phosphorylated when they are synthesized but this makes ³²P

end labeling reactions more difficult when screening plates). This reaction provides enough phosphorylated oligo for **many** ligation reactions.

1. 10X kinase buffer (provided by manufacturer)	5 μ l
2. unphosphorylated oligo, 20-25 μ g	x μ l
3. final ATP (pH 7.4) conc. of 1 mM	x μ l
4. T4 Polynucleotide Kinase (10 units)	1 μ l
5. Sterile water	(10-x) μ l
Total Volume:	50 μl

Incubate at 37°C for 1 hour. Heat to 65°C to kill enzyme [20 mins. for NEB kinase].

IV C. To concatemerize and hence elongate each oligonucleotide, ligate **complimentary pairs** of phosphorylated oligonucleotides (e.g. 5 μ g each of CAC_n and GTG_n) or a single phosphorylated oligonucleotide that is self complimentary (e.g. CG_n). (Alternatively, one can use T4 RNA ligase and individual oligonucleotides.) Addition of ATP to the ligation is optional but not usually necessary since it was added to the kinase reaction.

1. phosphorylated oligo(s) from IV. B (5 μ g)	x μ l
2. sterile water	x μ l
3. 10 x ligation buffer (supplied)	1.5 μ l
4. 1.5 Weiss unit T4 DNA ligase	1 μ l
Total Volume:	15 μl

Incubate overnight @ 16°C in water bath or thermocycler.

Set up multiple ligation reactions with the entire amount of kinased oligos. Ligated oligonucleotides will keep indefinitely at -20 while kinased oligos will slowly lose their 5' phosphates over time (months).

IV D. Run 3 μ l of the ligation in a 1.5% agarose gel to visualize the ligation products. Be sure to load a molecular weight size marker on the gel. A successful ligation is indicated by a band or smear of DNA that is much larger than the original oligonucleotides (e.g. several hundred base pairs).

IV E. To further increase the size of the oligonucleotide concatemer set up a self-priming PCR reaction in 50 μ l as follows:

1. Concatemerized oligonucleotide from step IV. D:	2 μ l
2. dNTP mix (8 mM):	5 μ l
3. PCR buffer (standard 10x, incl. MgCl ₂)	5 μ l
4. <i>Taq</i> Polymerase (3 units):	0.3 μ l
5. Sterile water:	37.7 μ l

Thermal profile: 94°C for 1 minute; 65°C for 1 minute; and 72°C for 2 minutes; 40 cycles. If MgCl₂ is not included in the PCR buffer add MgCl₂ to a final conc. of 1 or 1.5 mM and decrease amount of water.

IV F. Run 3 μl of these amplified repeats along with oligo ligation products and size marker on a 1.5% agarose gel to document the size and relative amount of the product. Again, successful amplification is indicated by a band or smear of DNA that is much larger than the original oligonucleotides and ligation products (e.g. several hundred to thousands of base pairs).

Pool PCR reactions in 100 μl aliquots and remove PCR components by precipitating with 1/10 volume 3 M NaOAc and 2 volumes ice cold 100% EtOH. Centrifuge at 12,000 rpm for 10 mins, wash with cold 70% EtOH and spin again for 4 mins. Air dry or Speedvac. Quantify via spectrophotometry or gel assay. **In addition, approximately 500ng should be kept for comparison with enriched products (see VI B).**

V. Hybridization of amplified inserts to amplified repeats.

V. A. Selection of inserts that contain microsatellites is accomplished by hybridization of the amplified inserts to amplified repeats bound to nylon filters. Different amplified repeats (e.g., $\text{CAC}_n/\text{GTG}_n$ and $\text{CATG}_n/\text{GTAC}_n$) can be pooled or used alone in hybridizations. If pooled, try to match repeats with similar annealing temperatures. Pooling is not recommended due to T_m differences. Treating repeats separately also gives indication of relative hybridization success for each repeat.

V. B. Denature 2 μg of amplified repeats (pooled or single) by alkaline addition or by heat denaturation. **Note that some brands of blotting membrane work poorly with alkaline denatured DNA. Read manufacturer's recommendations!** For alkaline denaturation with MSI MagnaCharge membrane add of 0.1 volume of 3 M NaOH and incubate at 60°C for 1 hour. Then neutralize with 1 volume of 6X SSC.

Cut 1 cm^2 pieces of membrane and label each square (DNA side up) with repeat sequence, species and individual. Labels are helpful if hybridization has to be repeated - one can reuse membranes without contamination and save lots of work. Wet membranes in di H_2O and soak in 6X SSC.

V. C. Spot at least 1 μg (more is better) of the denatured repeats onto filter. [Repeat each blot for optional insertless controls, see below.] Alternatively, use a dot blotter apparatus. Bake filters at 60-80°C for 1-2 hours and UV crosslink DNA to the filters. **Check membrane manufacturer's recommendations for DNA immobilization method!**

Rinse filters in denaturing solution (50 mM KOH/0.01% SDS) and then 6X SSC. This will wash away any oligo that did not bind (non-trivial amounts wash off) and will prevent lots of self-priming oligo amplification in the post-hyb PCR step.

V. D. Pre-wet filters in dH_2O for 10 or so minutes. Using clean forceps, place filters in a 1.5 mL screw-top microfuge tube DNA side toward the inside of the tube. Three or four filters for the same insert DNA should be placed in the same tube.

V. E. Prehybridize each filter in 1 ml of hybridization buffer (0.25 M Na_2HPO_4 , pH 7.2; 7% SDS; 1 mM Na-EDTA, pH 8.0; 1% BSA, fraction 5) at hybridization temperature for 2

to 6 hours. Pour out pre-hybridization solution and replace with 100 μ l of the same preheated hyb solution. Do the same for the optional insertless control filters.

V. F. Denature about 1 μ g of amplified inserts by heating to 100°C for 5 minutes and add directly to hybridization buffer while hot and mix with pipetor. No insert DNA is added to the insertless control filters!

V. G. Place screw-top tubes in rotating canister of a hybridization oven. Tubes should rotate along their long axis. Hybridize at 45 to 65°C overnight.

V. H. Wash unbound DNA from filters with 2.0 X SSC, 0.1% SDS. Multiple filters can be washed together in hyb tubes. Use 1 mL of solution and wash for 15 minutes at room temp, 30 minutes at hybridization temp (in my case 45°C). Heat wash solution in advance.

V. I. Remove and precipitate the bound microsatellite-enriched insert DNA from the filters. At this point filters should be put into individual 1.5 mL tubes.

1. Add 100 μ l of denaturing solution (50 mM KOH/0.01% SDS) and pipet solution over the filters 5-10 times at room temperature and let filter sit in solution for 5 min. **Save** each denaturing solution.
2. Place filter in new tube. Add 100 μ l of neutralizing solution (50 mM Tris-HCl, pH 7.5/ 0.01% SDS) and pipet solution over the filters 5-10 times and let filter sit in solution for 5 min. **Save** each neutralizing solution.
3. **Combine solutions** from the tubes in step 1 and 2. Add 1/10 volume of 3.0M Na-acetate (pH 7.0). Mix gently.
4. Add 2 μ l of **100 μ M SAULA primer** as carrier DNA.
5. Add 2 volumes ice-cold 100% ethanol and place in ultra-cold freezer for 30 minutes.
6. Spin at maximum velocity in refrigerated microfuge for 10 minutes to pellet DNA. Discard ethanol and rinse pellet in 400 μ l of 70% ethanol.
7. Discard 70% ethanol and speed-vac pellet until dry. Resuspend in 20 μ l of T.E.
8. Strip filters for reuse according to manufacturer's instructions.

VI. Amplification of microsatellite-enriched inserts and ligation into plasmid.

VI. A. The microsatellite-enriched inserts and the control, which may have oligo arrays which will self-prime, are reamplified in a 50 μ l PCR reaction with the SAULA primer as in III.D **except** that 5 μ l of post-hyb insert DNA is used. Run out 3 μ l of PCR product on 1.5% agarose to verify amplification and check control for amplification. If control shows amplification oligo arrays may have washed off filters or there may be template

contamination. Oligo contamination will make interpretation of the next step problematic so it can be skipped.

VI. B. Dot blot on a nylon filter: 500 ng of unenriched PCR product from step III.D and 500 ng of microsatellite-enriched PCR product [and the same volume of optional control amplification products as used for the enriched product].

1. Radioactively end-label original repeat oligos using polynucleotide kinase and $-^{32}\text{P}$.
2. Hybridize probe to filter at 45°C in hybridization solution (see V.e) overnight. Wash filter and expose to x-ray film. Enrichment is indicated by a darker dot for the enriched component versus the unenriched. If the dot of the insertless control is as dark as that of the enriched component, a false positive is implied (i.e., repeat arrays washed off the filter during hybridization and amplified).

VI. C. Remove the SAU linkers from the post-hyb amplified inserts to form cohesive ends for cloning. Multiple PCR reactions can be pooled to increase DNA amounts (scale digest as necessary).

1. Digest most of the PCR reaction with *DpnII* or isoschizomer to remove linkers.

a. post-hyb insert DNA	42 μl
b. 10x buffer	6 μl
c. 5 units of <i>DpnII</i> :	0.5 μl
d. sdi H ₂ O	11.5 μl

Incubate for 2 hours to overnight at 37°C . DO NOT heat kill enzyme.

2. Run an aliquot (3 to 5 μl) of the the digest on a 1.5% low melting point agarose gel to verify digestion. Also load an aliquot of undigested linker-insert DNA and linker DNA as size controls. Successful digestion is indicated by a bright band around 20 bp in length and a smear of insert DNA in the size range that was isolated initially.
3. If digestion of linkers is successful, remover linkers from insert DNA using a Qiagen Qiaquick PCR purification column according to manufacturer's protocol. Elute column in 30 μl T.E. This method of linker removal is preferred over Gelase (the NH_4COOH used to precipitate DNA after Gelase often inhibits PCR).

VI. D. Digestion and dephosphorylation of vector DNA:

1. Digest 10 μg of pBluescript with *BamHI* according to manufacturers conditions. Check for complete digestion by running digest on a 1.5 % agarose gel adjacent to uncut pBluescript.
2. Remove 5' phosphates from vector:

digested vector DNA	x μl
10x alkaline phosphatase buffer (supplied)	5.0 μl
sdi H ₂ O	y μl

alkaline phosphatase (CIP)	0.5 μ l
Total Volume: 50 μl	

Incubate for 1 hour at 37°C.

NOTE: NEB CIP is active in NEB BamHI buffer and can be added directly to vector digest. Use of NEB CIP at a concentration of 1/10 unit per pmole of DNA ends will be sufficient to dephosphorylate a 5' overhang (2 hour incubation) and the CIP can be heat killed in the presence of EDTA without need of phenol/chloroform.

3. Add 50 μ l of sterile TE to bring to a final volume of 100 μ l. Add to tube 200 μ l of phenol-chloroform, vortex and spin in microfuge for two minutes. Remove aqueous layer to a new tube and discard lower phenol-chloroform layer in waste.
4. Add to the aqueous supernatant 10 μ l (0.1 volumes) of 3 M sodium acetate (pH 7.0) and 200 μ l of ice-cold ethanol.
5. Freeze in ultra-cold for 20 minutes and spin in microfuge for 10 minutes to recover vector DNA pellet.
6. Wash in 70% ethanol as above and dry pellet in speedvac.
7. Resuspend in 75 to 100 μ l TE (depending on concentration desired).
8. Quantify both insert and vector amounts on 1.5 % minigel. Run uncut pBluescript vector for comparison.

VI. E. Ligation of vector and insert:

For complementary overhangs ligation proceeds well at 1:1 or 2:1 molar ratio of insert to vector DNA. About 100 ng of vector with about 100 ng of insert DNA was used successfully but conditions will vary and DNA amounts should be estimated from concentration information and molar end ratios.

1. Phosphorylate insert DNA just before ligation into plasmid. NEB kinase is active in NEB ligase buffer (which contains ATP). So if phosphorylation is done in ligation buffer we can later add ligase and plasmid directly and save some time and effort.

1. Sterile water	(8.5-x) μ l
2. 10X ligase buffer (NEB)	1 μ l
3. insert DNA	x μ l
4. T4 Polynucleotide Kinase (NEB)	0.5 μ l
Total Volume: 10 μl	

Incubate at 37°C for 1 hour. Heat to 65°C for 20 minutes to kill enzyme.

2. Now construct a ligation reaction using the kinase reaction as a base. More ATP is added to replenish that used up in the kinase reaction, giving a total concentration of 10 mM ATP.

phosphorylated insert DNA from above	10.0 μ l
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sterile water	5.5 μ l	
10x ligase buffer (NEB)		1.0 μ l
pBluescript (about 100 ng)	2.0 μ l	
10 mM ATP, pH 7.4	1.0 μ l	
T4 DNA ligase (NEB)		0.5 μ l
Total Volume:	20 μl	

Incubate overnight @ 16 °C in water bath or thermocycler. A ligation control with no insert is suggested.

VII. Transformation of competent *E. coli* cells and selection of colonies containing microsatellite inserts.

A. We used Stratagene Epicurian Coli® XL1-Blue supercompetent cells. Don't be cheap - buy supercompetent cells! Inoculate two to four plates per library (i.e., 100 and 200 μ l of transformation mixture). The following is from the Stratagene instructions.

1. Thaw supercompetent cells on ice.
2. Gently mix cells by hand. Aliquot 100 μ l of cells into a **prechilled** 15-ml Falcon 2059 polypropylene tube. (Note: using 50 μ l cells will work but usually requires 200 μ l of transformation mixture to be plated).
3. Add 1.7 μ l of β -mercaptoethanol (supplied with cells) to 100 μ l of bacteria to reach a final concentration of 25 mM.
4. Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.
5. Add 0.1-50 ng ligation mix to the cells and swirl gently.
6. Incubate on ice for 30 minutes.
7. Heat pulse the tubes in a 42 °C waterbath for **45 seconds**. The length of time of the heat pulse is critical for highest transformation efficiency.
8. Incubate the tubes on ice for 2 minutes.
9. Add 0.9 ml of preheated (42 °C) SOC medium (**not** LB) and incubate tubes at 37 °C for 1 hour with shaking at 225-250 rpm.
10. Use a sterile spreader to plate transformation mix on LB-agar/ampicillin plates in presence of IPTG/XGAL. Make sure ampicillin is fresh!!!
11. Place at 37 °C overnight.

B. Screening of library for positive clones containing microsatellites (see Sambrook et al.).

1. Make up thick LB/ampicillin plates with IPTG/XGAL to use as master plates. Draw grids on the agar side of the plates with a marker and ruler.
2. Using a sterile pipet tip pick white colonies on library plates and transfer to master plate within grid squares. Be sure to include a few blue colonies on each master plate to use as negative hybridization controls. Stab pipet tip into agar surface slightly as well as spreading cells on surface. Grow master colonies about 24 hours until they are large.
3. Use nylon hybridization membrane circles for colony lifts off master plates. Check protocol for brand of membrane. See Sambrook et al. for details. Use optional SDS soak

to reduce background. Bake filters for 1 hr at 80 °C and then UV crosslink. Filters can be stored at room temp between pieces of filter paper.

5. Prewet filters in H₂O and then prehybridize for at least 4 hours in 25 mL of hybridization solution (0.25 M Na₂HPO₄, pH 7.2; 7% SDS; 1 mM Na-EDTA, pH 8.0; 1% BSA, fraction 5).

6. Radioactively end-label individual or pooled repeat oligos using polynucleotide kinase and ⁻³²P. Labeling 20 pmoles of **each** oligo works well. Scale as necessary.

For five pooled oligos:

1. Sterile water		2.0 μl
2. 10X kinase buffer (NEB)	2.0 μl	
3. repeat oligos DNA (each 2.0 μl of 10pmol/μl)		10.0 μl
4. -dAT ³² P	5.0 μl	
5. T4 Polynucleotide Kinase (NEB)		1.0 μl
	Total Volume:	20 μl

Incubate at 37°C for 1 hour. 10 μl of this labeling reaction is enough for a tube of filters to be hybridized.

For single oligos:

1. Sterile water		1.0 μl
2. 10X kinase buffer (NEB)	1.0 μl	
3. repeat oligo DNA (each 2μl of 10pmol/μl)		5.0 μl
4. -dAT ³² P	2.5 μl	
5. T4 Polynucleotide Kinase (NEB)		0.5 μl
	Total Volume:	10 μl

Incubate at 37°C for 1 hour. 10 μl of this labeling reaction is enough for a tube of filters to be hybridized.

7. Pour out prehybridization solution and add 10 mL fresh hybridization solution. Add labeled oligos to hybridization solution. Hybridize probe to filter at 45-65°C overnight.

8. Wash filters for 15 minutes each:

- 1) once in 2X SSC, 0.1% SDS @ room temp
- 2) once in 2X SSC, 0.1% SDS half-way to hybridization temp
- 3) once or twice in 2X SSC, 0.1% SDS at hybridization temp
- 4) rinse filters in 0.2 X SSC at room temperature to remove SDS.

Heat wash solutions in advance. 2X SSC, 0.1% SDS can be used as a less stringent final wash or the last stringent wash can be skipped. Expose to x-ray film.

9. Clones that contain microsatellites are usually much darker on the autoradiograph than those that do not. Inserts from positive clones are PCR amplified for direct sequencing (recommended) or cells are miniprep for plasmid isolation (not recommended because it is too much work). Miniprep protocols for plasmid isolation can be found in Sambrook et al. 1989 and Applied Biosystems "Bulletin 18".

C. Amplification of insert DNA.

Pick a few cells from a positive colony, boil them to release the plasmids and then amplify the insert with flanking plasmid primers such as T7 and T3.

1. Label 500 μ l microfuge tubes and fill them with 100 μ l T.E.
2. Using a sterile pipet tip, carefully pick cells from the center of a positive colony. Wash off the cells into the T.E by flicking the pipet tip in the solution.
3. Place the tubes with cells and T.E in a 100°C heat block for 10 minutes.
4. Spin tubes for 5 minutes at max speed to pellet cells.
5. Use 1 μ l of this solution as template in a 50 μ l PCR reaction (remember to include a blue colony as a control for polylinker amplification).
6. Run PCR products on a 1.5% gel to check amplification.
7. Clean up PCR reactions with QiaQuick PCR purification preps and sequence using T7 and/or T3 as primers.

VIII. Sequencing of positive clones and primer design.

A. We typically sequence plasmids or insert amplification products using a standard double-stranded protocol. We had good success with both manual (Sequenase 2.0, U.S. Biochemical) and automated (*Taq* Cycle Sequencing, Applied Biosystems Incorporated) methods. We refer the reader to these vendors or others for detailed protocols.

B. Primers are designed from sequences flanking the microsatellite arrays identified within the inserts. Occasionally a microsatellite array begins on the edge of the plasmid, or is incomplete or too large for continued development. In addition, some clones contain the same insert. In general only a fraction (>30%?) will yield useful microsatellite primers.

C. Design rules are available from a number of sources (e.g. Hoelzel and Green 1992). Free software such as Amplify for the Mac is useful to design primers.

D. Primers must be tested and optimized in order to guarantee faithful and consistent amplification. In addition, microsatellites should be assessed with pedigreed families to ensure Mendelian inheritance and independent assortment.

Large repeats that lack sufficient flanking sequence to design primers:

Sequencing often reveals large numbers of repeats that lack enough flanking region to design a primer. In this case it is possible to extend the known sequence and get enough flank for a reliable primer. See for example Ochman, H., M. M. Medhora, D. Garza, and D. L. Hartl. 1990. Amplification of flanking sequences by inverse PCR. Pp. 219-227 in M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White (eds.). PCR Protocols. A Guide to Methods and Applications. Academic Press, San Diego.

Not So Standard Solutions:

T.E: 10mM Tris-HCl, pH 8.0; 0.1mM EDTA, pH 8.0 (reduced conc. of EDTA will not inhibit PCR reactions)

Appendix 1

The molecular weight of double stranded DNA,

$$MW_{ds} = \text{\#base pairs}(650 \text{ daltons/bp})$$

The moles of **ends** of double stranded DNA = $2(\text{grams DNA}) / (\text{MW in daltons})$

For DNA that is of variable length such as a genomic digest you must estimate the average base pair length of the entire sample. For example, a digest that is size selected for 200 to 800 dp fragments has an average length of about 500 bp.

a 500 bp fragment is $500(650) = 325,000$ daltons

1 μg of this genomic DNA has

$$2(1 \times 10^{-6} \text{ grams}) / 325,000 \text{ daltons} = 6.154 \times 10^{-12} \text{ moles of ends}$$

For DNA of fixed length such as oligonucleotides calculate the MW in daltons:

a 20 bp oligo is $20 \text{ bp}(650 \text{ daltons/bp}) = 13,000$ daltons

A 1:1 molar end ratio for genomic DNA ends to oligo DNA ends will require 6.154×10^{-12} moles of ends of oligo in solution. So for a 20 bp oligo

$$Y \text{ grams oligo} = (6.154 \times 10^{-12} \text{ moles of ends})(13,000 \text{ daltons}) / 2 = 4.0001 \times 10^{-8} \text{ grams or } 40 \text{ ng}$$

In general

$$Y \text{ grams} = (\text{moles of ends})(\text{ratio factor})(\text{MW in daltons}) / 2$$

For a cohesive termini ligation the ratio factor is 1 or 2. In the case of a blunt end ligation the ratio factor for the oligo would be between 40 and 100.

NEB recommends a DNA concentration of 0.1 to 1 μM of 5' termini. USB says that "for each 10 μl of reaction volume, a combination of 20-40 ng vector and a three to ten-fold excess of foreign DNA will produce an adequate yield of recombinant genomes for most cloning purposes."

Additional restriction enzyme/linker combinations

Choice of enzyme used to digest genomic DNA should be based on cut sites available in the plasmid polylinker, on restriction surveys of the genomic DNA to be used and sequence of microsatellite repeats desired. The object is to get a large proportion of the genome into the 900-200 bp size range with a complimentary overhang. Four-cutters will cut most frequently but some six-cutters may give enough digestion to be used. A restriction enzyme with a palindromic overhang sequence must be used unless you are willing to synthesize TWO overhang linkers and deal with the resulting complications (e.g. double digests to remove linkers).

Taq I (TCGA/AGCT) provides a 5' - CG -3' overhang that can be used with the Cla I site in the pBS SK+/- polylinker. For example:

5' -GCG GTA CCC GGG AAG CTT GG -3' with 5' - CGANNNN -3'
 3' -CGC CAT GGG CCC TTC GAA CCGC -5' TNNNN

and

5' - NNNNT -3' with 5' - CGCC AAG CTT CCC GGG TAC CGC -
 3'
 3' - NNNNAGC -5' 3' - GG TTC GAA GGG CCC ATG GCG -
 5'

In this case the "SAULA" linker can be used as with Dpn II but the "SAULB" linker must be synthesized to have a CG overhang (TaqB).

1. SAULA: 5' - GCG GTA CCC GGG AAG CTT GG -3'
2. TaqB: 5' - CGC CAA GCT TCC CGG GTA CCGC -3'

Additional restriction enzyme/linker combinations can be constructed using compatible cohesive end charts in the NEB and Stratagene catalogs.