SOLTIS LAB MICROSATELLITE CAPTURE PROTOCOL
Adapted from the UF ICBR Molecular Markers Workshop
Last updated: 20 September 2004

References:

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Kijas JMH, JCS Fowler, CA Garbett, and MR Thomas. 1994. Enrichment of microsatellites from the citrus genome using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles. Biotechniques 16(4): 657-660.

Genomic DNA Digestion

This first step in the microsatellite capture protocol uses restriction enzymes to cut genomic DNA into smaller fragments. We will be using *Sau*3A I, which has a recognition sequence of GATC.

REMEMBER: Always keep restriction enzyme on ice!

CLEARLY LABEL EACH TUBE WITH THE SAMPLE NAME, THE DATE, AND SOME DESIGNATION THAT THIS IS A DIGESTION.

Reagent	Amount
Genomic DNA (~0.24 µg/µl)	21.5 µl
10X *Sau* 3A I buffer b	2.5 µl
10 U/µl *Sau* 3A I enzyme	1.0 µl
TOTAL	25.0 µl

Incubate at 37º C overnight in water bath.

Prepare a 1.5% agarose gel using TBE buffer. You will run 5 µl of undigested genomic DNA and 5 µl digested genomic DNA to test the success of your digestion. Use a size standard with a large range of bands (such as the λ Pst I marker). Your digested DNA

should be a long smear that is somewhat smaller than your undigested DNA, approximately between 400 bp and 1500 bp in length.

DNA purification and size fractionation using Chroma Spin columns

The purpose of this step is to extract fragments greater than 400 bp in length from the digested DNA. Using fragments smaller than this will make primer design difficult.

1. Add 20 µl of TE buffer to 20 µl of digested genomic DNA for a total volume of 40 µl. The chroma spin columns have a volume requirement, so be sure to keep a total volume of 40 µl or more.

2. Remove the Chroma Spin column from the protective plastic bag, invert it several times to resuspend the gel matrix completely.

3. Before using Chroma Spin column, label the **side** of each one with the appropriate sample name; these columns will be reused. Holding the Chroma Spin column upright, grasp the break-away end and snap off. Place the end of the spin column into one of the 2 mL microcentrifuge (collection) tubes provided. You do not need to label the collection tubes. **Remove all caps and save them for later**.

4. Centrifuge for 5 minutes at 700 g in IEC centrifuge. After centrifugation , the column matrix will appear semi-dry. This step purges the equilibration buffer from the column and re-establishes the matrix bed.

5. Remove the spin column and the collection tube from the centrifuge and discard the collection tube and column equilibration buffer.

6. Place the spin column into a new 1.5 mL microcentrifuge tube labeled with the sample name. Carefully and slowly apply the digested genomic DNA mixture to the center of the gel bed's flat surface. Do not allow any sample to flow along the inner wall of the spin column.

7. Centrifuge for 5 minutes at 700 g in IEC centrifuge.

8. Remove the spin column and collection tube from the centrifuge. Your purified sample is at the bottom of the collection tube. **DO NOT DISPOSE OF THE COLUMNS YET! YOU WILL USE THEM AGAIN.** Add 1 mL of TE buffer to the empty column to keep the resin bed wet until the next use.

9. Use the Promega Wizard SV PCR clean-up system to concentrate your purified samples. Follow the directions provided with the kit. Elute samples in 20 µl of buffer.

BE SURE TO CLEARLY LABEL YOUR FINAL TUBES WITH THE SAMPLE NAMES, THE DATE, AND SOME DESIGNATION THAT THIS IS SELECTED DIGESTS.

Ligation of fractionated genomic DNA to *Sau* 3A I linkers and library amplification

This step adds a linker to both ends of your DNA fragments. A linker is a piece of double stranded DNA that is designed for "tagging" your fragments. Since the sequence of your fragments is unknown, by adding a known sequence, you can use PCR to amplify your fragment. We will be using the following *Sau* linkers:

*Sau*3AI-L-A: 5'-GCGGTACCCGGAAGCTTGG-3'
Sau-L-B: 5'-GATCCCAAGCTTCCCGGGTACCGC-3'

Preparation of the *Sau* linkers

Reagent	Amount
10X T4 Ligase buffer*	5 µl
Unphosphorylated *Sau*-L-B linker	25µg (3.5 µl of 100 µM)
T4 Polynucleotide kinase (5-10U/µl)	3µl
Autoclaved dd H_2O	Bring total to 50µl

1. Aliquot the solution into PCR tubes and incubate at 37º C for 1 hour, followed by 68º C for 10 minutes. Cool to room temperature.

2. Add 3.5 µl (100 µM) of *Sau*-L-A linker and let sit at room temp for 5 minutes.

3. Dilute to 0.5µg/µl

* Use the T4 ligase buffer rather than the T4 polynucleotide kinase (PNK) buffer since the PNK enzyme needs ATP, which is included in the T4 ligase buffer, and PKN is 100% active in the ligase buffer.

REMEMBER: Always keep the T4 DNA Ligase on ice!

CLEARLY LABEL EACH TUBE WITH THE SAMPLE NAME, THE DATE, AND SOME DESIGNATION THAT THIS IS A LIGATION.

Reagent	Amount
Sau 3A I linker (0.5 µg/µl)	2.00 µl
Digested/fractionated genomic DNA	10.00 µl
Water	2.00 µl

10 X Ligase buffer	2.0 µl
10 X BSA solution	2.0 µl
T4 DNA Ligase	2.0 µl
TOTAL	20.0 µl

Mix well by pipeting and incubate on bench top overnight.

Removal of excess *Sau* 3A I linker from genomic DNA by fractionation using Chroma Spin columns

USE THE SAME COLUMNS USED IN DNA PURIFICATION AND SIZE FRACTIONATION.

CLEARLY LABEL TUBES WITH SAMPLE NUMBER, DATE, AND SOME DESIGNATION THAT THIS IS FRACTIONATED LIGATED DNA.

1. Add 20 µl of TE buffer to ligation reaction (total volume = 40 µl)

2. Spin the previously used Chroma spin column to remove the TE buffer and pack the resin bed. Centrifuge for 5 minutes at 700 g. After centrifugation, the column matrix will appear semi-dry. This step purges the equilibration buffer from the column and re-establishes the matrix bed.

3. Remove the spin column and the collection tube from the centrifuge and discard the collection tube and column equilibration buffer.

4. Place the spin column into a new 1.5 mL microcentrifuge tube labeled with the sample name. Carefully and slowly apply the ligated digested genomic DNA mixture to the center of the gel bed's flat surface. Do not allow any sample to flow along the inner wall of the spin column.

5. Centrifuge for 5 minutes at 2800 RPM.

6. Remove the spin column and collection tube from the centrifuge. Your purified sample is at the bottom of the collection tube.

Amplification of linker-ligated fragments ("Whole genome PCR library")

CLEARLY LABEL TUBES WITH SAMPLE NUMBER, DATE, AND SOME DESIGNATION THAT THIS IS THE WHOLE GENOME LIBRARY.

Reagents	Amount
Water	27.0
10 X Buffer	5.0
25 mM $MgCl_2$	3.0
2.5 mM dNTPs	2.0
10 µM *Sau* 3A I Primer	2.5
5 U/µl Taq	0.5
Fractionated Ligated DNA	10.0
TOTAL	50.0

PCR Profile is as follows:

"SSR-PCR1"

Temperature	**Duration**
72º C	2 minutes
94º C	3 minutes
94º C	1 minute
68º C	1 minute
72º C	2 minutes
GOTO 2	25 times
72º C	10 minutes
4º C	HOLD

Microsatellite library enrichment using Vectrex Avidin D

Denaturation of genomic PCR library (400 – 1500 bp fragments)

YOU WILL NEED TO PRE-HEAT THE 2X HYBRIDIZATION BUFFER AT 48º C FOR THE FOLLOWING STEPS.

1. Aliquot 15 µl of PCR 1 product into a clean tube.

2. Denature the whole genome library by incubating the tube at 98º C for 10 minutes, immediately followed by a quick chilling on ice for at least 2 minutes.

3. While denaturation is taking place, prepare the probe solution by mixing 0.5 µl of biotinylated oligoprobe (~0.5 µg, single stranded) with 19 µl of 2X hybridization buffer (2XH).

Recipe for 2XH	For 500 mL
1 M Sodium phosphate, pH 7.4	78.00 g $NaPO_4$
1% SDS	5 g SDS
1%BSA	5 g BSA
	Bring vol up to 500 mL with water

Hybridization

1. At the end of denaturation, add the probe solution to the denatured whole genome library.

2. Allow hybridization to take place overnight by incubating at 48º C while slowly rotating samples.

Preparation of VECTREX Avidin D Matrix

1. While hybridizing, transfer 20 µl of a 1:1 slurry of VECTREX Avidin D matrix (BE SURE TO VORTEX THOROUGHLY) into a clean tube.

2. Wash VECTREX Avidin D matrix as follows:

    a. Centrifuge for 15 seconds at 14,000 rpm

    b. Pipette out and discard supernatant carefully

    c. Add 50 µl 1X TBST to the tube

Recipe for 1X TBST	For 250 mL
0.1 M Tris pH 7.5	25 mL 1M Tris
150 mM NaCl	7.5 mL 5 M NaCl
0.1% Tween	250 µl Tween
	Bring vol up to 250 mL with water

    d. Vortex and centrifuge as before

    e. Discard supernatant and repeat 1X TBST wash

3. Block nonspecific binding of DNA to the VECTREX Avidin D matrix by resuspending with 50 µl TBST containing 100 µg/ml of sheared salmon testes DNA.

4. Incubate for 30 minutes at room temperature with continuous mixing using the vortexer.

5. Remove unabsorbed salmon testes DNA by washing matrix three times with 100 µl 1X TBST as before (i.e., centrifugation followed by careful discarding of supernatant with pipette).

Capturing of biotinylated probe hybridized to repeat-containing fragments

1. At the end of hybridization, add 720 µl of TBST to the hybridization mixture and transfer the entire volume into the tube containing the VECTREX Avidin D matrix preparation.

2. Incubate at 37º C for 30 to 60 minutes with continuous rotation. DO NOT INCUBATE FOR MORE THAN 60 MINUTES.

3. Spin tube to pellet.

4. Transfer supernatant to new tube. Label "impoverished DNA".

5. Wash resin as before (i.e., resuspending, centrifuging and discarding supernatant) using the following sequence:
   - Once with 500 µl TBST, room temperature (always mix briefly before centrifuging).
   - Once with 500 µl TBST, 65º C, 10 minutes with mixing.
   - Once with 500 µl 0.1X TBST, 65º C, 10 minutes with mixing.

6. Elute captured fragments by resuspending matrix in 200 µl water and incubating at 65º C for 30 minutes with mixing.

7. Centrifuge for 30 seconds and transfer supernatant carefully into clean microcentrifuge tube properly labeled with the sample name, date, and "select DNA". You can now throw away the VECTREX tubes.

PCR amplification of genomic fragments enriched for $(CA)_n$ repeats

Reagents	**Amount**	**Final Conc.**
Water	27.0	-
Eluted DNA fragments	10.0	-
10X PCR Buffer	5.0	1X
25 mM $MgCl_2$	3.0	1.5 mM
2.5 mM dNTPs	2.0	100 µM each
10 µM *Sau* 3A I Primer	2.5	0.5 µM
5.0 U/µl Taq	0.5	2.5 U/50 µl
TOTAL	50.0	

PCR Profile is as follows:

"SSR-PCR1"

Temperature	**Duration**
94º C	3 minutes
94º C	1 minute
68º C	1 minute
72º C	2 minutes
GOTO 2	25 times
72º C	10 minutes
4º C	HOLD

Run a test gel on the PCR products. Assuming successful PCR, following with cloning using the TA Topo kit. HALF REACTIONS MAY YEILD MORE DESIRABLE RESULTS FOR SUBSEQUENT COLONY LIFTS.

Recipe for Agar plates

500 mL water
5 g yeast
2.5 g triptone
2.5 g NaCl, pH to 7.3 using NaOH
7.5 g agar
(for 1 L recipe use 0.05 g ampicillin)

After combining ingredients in bottle, no need to mix; just autoclave.

Colony screening / direct lifts

ALWAYS USE GLOVES WHEN WORKING WITH MEMBRANES.

1. If you have not done so, incubate plates in the refrigerator for at least 1 hour. This will keep agar from sticking to the membranes in following steps.

2. While plates incubate, prepare the following materials for treatment of the membranes:

    a. Place one piece of Whatman filter paper into each of 5 Pyrex dishes.

    b. Pour the following solutions into the designated dishes. Use just enough solution to saturate the Whatman paper.

Dish 1: 10% SDS

Dishes 2 and 3: Denaturing solution (0.5 M NaOH, 1.5 M NaCl)
NaOH 20 g/L
NaCl 87.66 g/L

Dish 4: Neutralizing solution (0.5 M Tris, pH 7.4; 1.5 M NaCl)
Tris 60.57 g/L
NaCl 87.66 g/L
~ 20 mL HCl to pH

Dish 5: 2X SSC – DO NOT NEED WHATMAN PAPER

3. Using a pencil, label a circular nylon hybridization membrane opposite to the side that will be in contact with the bacterial colonies. The label should include your initials, the plate designation, and the date.

4. Retrieve the plate(s) from the refrigerator and place a nylon membrane onto the surface of the appropriate plates as follows: Hold the membrane at opposite edges with forceps, bending it slightly into a 'U'-shape, and then lower the membrane until the fold makes contact with the center of the plate. Lower the edges until the entire membrane is evenly wet.

5. Reference the membrane with the underlying agar by puncturing both with a pointer (that has been heated) at three asymmetrically located sites. Pick up the plate and view it from below. Use a marker to mark the puncture sites on the bottom of the plate.

6. Leave the membrane(s) on the agar at room temperature for 5 minutes and then carefully peel them off of the plates.

7. Place the first membrane, COLONY SIDE UP, onto the Whatman paper in dish 1 for a 5 minute treatment. Several membranes can be treated simultaneously.

8. Use forceps to transfer each membrane to dish 2 for a 5 minute treatment. Repeat this process until each membrane has undergone a 5 minute treatment in each of the five dishes. Place membranes on clean Whatman paper after the final treatment.

9. Cross-link the DNA on the membrane(s) by placing them in a UV. Use the program appropriate for cross-linking a damp membrane ("optimal crosslink").

10. Save the agar plates at 4º C until needed to recover the microsatellite-positive clones.

Hybridization with $(CA)_n$ probe and chemiluminescent detection

THIS PROCESS WILL BE DONE USING THE HYB-OVEN TUBES. You will need the following recipes:

Wash I	**For 250 mL**
Quick Light Wash Component A	10 mL
Quick Light Wash Component B	62.5 mL
dH_20	177.5 mL

Wash II	**For 250 mL**
Quick Light Wash Component A	1 mL
Quick Light Wash Component B	12.5 mL
dH_20	236.5 mL

BE SURE TO PRE-HEAT THE HYB-OVEN, WASH I, WASH II, AND THE QUICK LIGHT HYBRIDIZATION SOLUTION AT 55º C.

1. Place the membrane in a clean hyb-oven tube.

2. Add 40 ml of Wash II pre-warmed at 55º C. Incubate at 55º C in the hyb-oven with constant rotation for 10 minutes.

3. Pour Wash II in the sink and add 6 mL of the probe solution (made by mixing 0.5 µl of Quick Light probe solution in 6 ml of Quick Light hybridization solution). Incubate at 55º C in the hyb-oven with constant rotation for 20 minutes.

4. Transfer membranes to clean hyb-oven tube. Add 40 ml of Wash I prewarmed at 55º C. Incubate at 55º C in the hyb-oven with constant rotation for 10 minutes.

5. Discard Wash I and add a fresh 40 ml of Wash I. Incubate at 55º C in the hyb-oven with constant rotation for 10 minutes.

6. Discard Wash I and add 40 ml of Wash II. Incubate at 55º C in the hyb-oven with constant rotation for 10 minutes.

7. Pour off Wash II and add 25 ml of 1X Quick-Light (Lifecodes) buffer solution. Wash membrane briefly at room temperature.

8. Pour off the Quick-Light buffer and repeat this wash three more times for a total of four washes.

9. Remove the membrane(s) using forceps and allow excess buffer to drain off. Place membranes inside clear sheet protector, but do not close the protector.

10. Spray membranes lightly and evenly with Lumi-Phos 480 dioxetane. Carefully close sheet protector, taking care to smooth out bubbles on or around the membranes.

11. Place sheet protector with membranes in X-ray cartridge with film for overnight exposure. Develop film the following morning.

12. Inspect X-ray film for microsatellite-positive clones. Using a light box, line up agar plates with X-ray and mark positive colonies on plates.

13. Positive colonies should be grown overnight in 2 ml luria broth using tubes with membrane caps. Incubation should occur in a shaker set at 37º C.

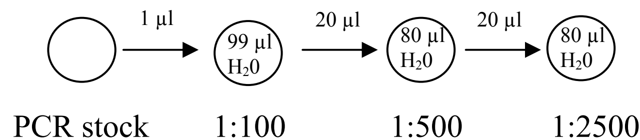
14. Spin down colonies for 5 minutes at maximum speed. Isolate DNA using Qiaprep spin miniprep kit (250).

Testing selection-hybridized DNA fragments for microsatellite enrichment

YOU MAY SKIP THIS STEP AND GO DIRECTLY TO PCR (SKCLONE).

Dot blot preparation

1. Prepare serial dilutions (1:100, 1:500, and 1:2500) from the enriched library (mini-prep product from last step).



2. Use a pencil to label a small piece of nylon membrane (ALWAYS WEAR GLOVES WHEN HANDLING MEMBRANES). Label the membrane as follows:

	PCR	1:100	1:500	1:2500
1				
2				
3				
4				
5				
6				

3. Dot 1µl of each dilution onto the membrane in the designated squares. Keep track of the side of the membrane onto which the DNA was blotted.

4. Prepare the following materials for the treatment of the membranes:

    a. Place one piece of Whatman paper into each of four pyrex dishes

    b. Pour the following solutions into the designated dishes. Use just enough solution to saturate the Whatman paper.

Dishes 1 and 2: Denaturing solution (0.5 M NaOH, 1.5 M NaCl)

Dish 3: Neutralizing solution (0.5 M Tris, pH 7.4; 1.5M NaCl)

Dish 4: 2X SSC

5. Transfer the membrane (DNA SIDE UP), onto the Whatman paper in dish one. Leave the membrane there for 5 minutes.

6. Using forceps, transfer the membrane onto the Whatman paper in dish two, leaving it there for 5 minutes.

7. Using forceps, transfer the membrane onto the Whatman paper in dish three, leaving it there for 5 minutes.

8. Using forceps, transfer the membrane onto the Whatman paper in dish four (the final dish), leaving it there for 5 minutes.

9. Using forceps, transfer the membrane to a clean piece of Whatman paper.

10. Cross-link the DNA on the membrane by placing it in a UV chamber (GS Gene Linker, Bio-Rad Laboratories, Inc., Hercules, CA). Use the appropriate program for cross-linking a damp membrane (program C3).

11. After cross-linking, follow the procedure for hybridization with $(CA)_n$ probe and chemiluminescent detection as outlined above. The final product will be an X-ray film of the positive dot blots. DNAs with multiple positives should be appropriate for sequencing.

Sequencing microsatellite-positive clones

AGAIN, YOU MAY SKIP THIS STEP AND PROCEED DIRECTLY TO PCR (SKCLONE).

1. First, check fragment sizes by digestion:

Reagents	Amount
Water	6.0
ECORI Buffer	1.0
ECORI	1.0
DNA Template	2.0
TOTAL	10.0

Digest samples at 37º C for 1 hour.

Run digests on 1.5% Agarose gel using entire reaction and 2µl of 6X loading dye.

2. PCR the samples using 1:10 dilutions (in water) and the "SKCLONE" program:

Reagents	Amount
10 X Soltis Buffer	5.0
2.0 mM dNTP	5.0
10 µM F M13	2.5
10 µM R M13	2.5
Taq	0.5
Water	29.5
DNA (1:10)	5.00
TOTAL	50.00

Run PCR products on test gel using hyperladder.
Continue with cycle-sequencing for good products.