Construction and Screening of a Subgenomic Library Highly Enriched for Microsatellite Regions

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Introduction

This document describes what we believe to be one the fastest and easiest way to isolate repeat microsatellite regions from which PCR primers suitable for use in population genetics and behavioral ecology can be developed. This method is also one of the most cost effective approaches, and the purchase of the reagents needed for building and screening one library will supply sufficient reagents for ten or more libraries. This method is a modification of Tenzer *el al.*'s (1999) approach to finding microsatellites, with subsequent modifications implemented by Barbara Gautschi and Trent Garner of the Institut für Umweltwissenschaft at the Universität Zürich-Irchel (Garner et al. 2000; Gautschi et al. 2000), and implemented in the development of microsatellites for *Arapaima gigas* (Farias et al. 2003), *Cynoscion acoupa* (Farias et al. 2006), *Paracheirodon axelrodi* (d'Assunção 2006), *Batcris gasipaes* (Rodrigues et al. 2004), *Podocnemys unifillis* (Fantin et al. 2007) as well as numerous other still unpublished species (*Symphysodon discus*, *Austrofundulus limnaeus*, *Brachyplatistoma filamentosus*, *Potamotrygon motoro*, *Melanosuchus niger*, *Inia geoffrensis*, *Trichechus inunguis*). In general, if all steps work, you can go from tissue to several hundred positive clones in about a week, depending on the type of repeat you wish to screen for. This approach is incredibly robust, and it's only drawback is that redundant clones are occasionally generated during the PCR steps of the enrichment process. It is interesting to note that given the same set of conditions, some species have very high percentages (~90%) of clones with perfect and near perfect repeats, while other species have low percentages (50% or less). This is not a methodological artifact, and most like reflects the relative frequency of microsatellite sequences in the genome. We have purposely described steps in what molecular-types would find excruciating detail, as the interest in finding and applying microsatellites does not lie strictly with molecular biologists and it is ridiculous that those less familiar with the cookbook methods of molecular biology should be bogged down by technical details and held back from addressing the really interesting questions that they wish to attack.

Before you start

You will need general lab equipment, such as a refrigerator and freezer, a PCR machine, a UV trans-illuminator for visualizing DNA on ethidium bromide-stained agarose gels, a bench top 13,000+ rpm centrifuge that accepts 1.5 ml Eppendorf tubes, electrophoresis apparatus for

agarose gels, pipettes of various sizes, a hybridization oven and optionally also a spectrophotometer and a water bath. You will also need general lab consumables such as sterile Eppendorf tubes, sterile pipetting tips of various sizes, sterile gloves, sterile 1.5 and 50 ml tubes, sterile PCR tubes and plates, etc. You will also need the various chemicals, kits and reagents listed below. It is highly recommended that you first attempt this technique with someone who is familiar with many molecular lab techniques. This will save you money, and more importantly, time and effort.

Methodology

Tissue sample and extraction[1]

A single candidate sample of tissue is required for extracting DNA that will be used for building the DNA library. Although this requires very little tissue (just a few milligrams), it is best to avoid tissue types that contain very little DNA or contain things that make extraction more difficult (e.g., bone, excessively fatty tissue). Also, it is relatively important that once the tissueis sampled, it is either extracted immediately or stored in a manner that will eliminate the potential degradation effects of endogenous restriction endonucleases. Freezing tissue in liquid nitrogen, in a -80°C freezer, storing tissue in 100% ethanol, or storage in a DMSO buffer are all viable options. Since both the development of an enrichment library and application of PCR primers requires small amounts of DNA, it should never be necessary to kill a macro-organism for research. In many studies, blood has been extracted and used to isolate DNA without harm to the animal. Shed skin from whales and reptiles, hair from mammals, and feathers from birds may also be used, but these tissues generally require very specific extraction methods and often provide DNA suitable for PCR, but unsuitable for library construction.

Once you have your tissue sample, extract the DNA using any of the standard DNA extraction methods. A good one is the protocol for isolating DNA from mammalian cells outlined in Sambrook *et al*. (1989). An important point in this protocol is to do the phenol extraction two times, and to cut the cut the tip of your blue pipette tip (use a sterile razor and cut on a clean and sterile piece of filter paper) before you remove the aqueous layer. This widens the bore of the tip and will reduce the shearing affect of pipetting. The objective is to obtain good quantity of high molecular and very pure DNA.

Standard phenol/chloroform method is the preferred method, but not the only method of extracting DNA. Chelex extraction is not clean enough for this step plus it does not prevent further degradation of DNA. Spin column technology (e.g. Qiagen) produces very pure DNA, but also shears high molecular fragments. CTAB extraction method may be best for high polysaccharide tissues such as plant material and snails.

Following is a brief outline of the Sambrook *et al*. (1989) DNA extraction method:

1 This DNA extraction protocol is for high molecular DNA. For basic microsatellite development you can just use Qiagen DNA extraction columns.

Materials needed
1 M Tris buffer, pH 8.0, autoclave sterilized
20% SDS (Lauryl sulfate), filter sterilized
10 mg/ml RNAase
0.5 M EDTA, pH 8.0 autoclave sterilized
ddH_2O
20 mg/ml proteinase K
pH 8.0 buffered (equilibrated) phenol
24:1 chloroform/isoamyl alcohol stored at -20°C
3 M sodium acetate (pH 4.6)
100% ethanol stored at -20°C
70% ethanol

In a 10 ml tube, combine 2.0 ml of EDT A, 7.63 ml ddH_2O, 100 µl Tris, 250 µl SDS and 20 µl of RNAase. This extraction buffer contains more RNAase than recommended by Sambrook, but ensures that RNA is almost completely eliminated from the extraction. Also, this buffer is stable on the bench top for several weeks. Cut, mash or grind tissue sample (1 gram or so), taking care to keep the sample from being contaminated with non-target DNA. In an Eppendorf tube, aliquot 500 µl to 1 ml of extraction buffer (depending on how much tissue you have) and add the macerated tissue (note: don't macerate tissue excessively, as this will also serve to degrade chromosomal DNA. The mashing/grinding step is done simply to allow the extraction buffer access to cells hidden under keratinized skin layers and to increase the total cellular surface area exposed to the extraction buffer). Incubate the tube in a water bath at 37°C for one hour. After one hour, add 5 ml of proteinase K (again excessive by Sambrook standards, but it also gives very clean DNA) and incubate for a further four or five hours at 55°C. Take care that the increase in incubation temperature does not cause the lid of the Eppendorf tube to pop open and allow contaminants in (cheap tubes tend to pop often).

After incubation, add an equal volume of equilibrated phenol (500 µl to 1 ml) to the tube and gently mix for ten minutes by inverting the tube. Phenol is a nasty organic solvent, so do all phenol steps in a fume hood and wear gloves. Eppendorfs often leak when phenol is involved. Make sure your lab has adequate means to deal with organic solvent wastes. After mixing, centrifuge the tube for 5 minutes at full speed (13,000 rpm), and then CAREFULLY pipette the aqueous (top) layer off and put in a clean Eppendorf. This is the trickiest step, as the organic (bottom) layer that contains all the unwanted cellular debris, and the whitish interface between the two layers that contains all the proteins degraded by the proteinase K will be sucked up with the aqueous phase if you pipette too aggressively. Watch carefully for the interface to ‘jump’ at the pipette tip while you aliquot off the aqueous layer. At this point it is better to be wasteful rather than greedy so that you will end up with good, clean DNA and not a lot of garbage in your sample. A further note: cut the tip of your blue pipette tip (use a sterile razor and cut on a clean and sterile piece of filter paper) before you remove the aqueous layer. This widens the bore of the tip and will reduce the shearing affect of pipetting.

After you have moved the aqueous layer to a new tube, repeat the phenol extraction step with a suitable volume of phenol and again pipette off and save the aqueous as described above. This step may need to be repeated again if a white interface layer is obvious during the first repetition. Once you have moved the aqueous layer to a clean tube for the final time, add an equal volume of chloroform/isoamyl alcohol to the tube, gently invert the tube a few times, spin at high speed for only two minutes, and again remove the aqueous layer. Clip your pipette tip again, but you will notice that it is much easier to pipette off the aqueous laxer with chloroform (you can be more greedy here). To this final aqueous extract, add 1/10 th volume of 3 M sodium acetate and two volumes of 100% ethanol. Put the tube in the -20°C freezer overnight. The next morning, centrifuge the tube at full speed for 30 minutes, pipette off the ethanol, taking care not to remove that whitish or translucent pellet at the bottom of the tube, and add 200 µl of 70% ethanol. Finger vortex the tube gently a few times, spill again at high speed for five minutes, and remove the ethanol layer again. Dry the pellet for a few minutes in a SpeedVac concentrator, and resuspend in 20 µl of ddH_2O. Let the pellet of DNA dissolve into the water by incubating the tube at about 50°C for ten or so minutes.

Use a spectrophotometer to determine the DNA concentration of this extraction, or simply approximate the amount of DNA in your extraction by running it against a molecular size standard such as Lambda/HindIII marker (this will also simultaneously show you the quality of the extracted DNA). Dilute your DNA to100 ng/µl.

Digesting and size fractioning the DNA

Materials needed:
MboI (Sau3A) restriction enzyme and l0x buffer R (Fermentas)[2]
Agarose (Fisher Low EEO)
Lambda/HindIII DNA molecular marker (Fermentas)
Lambda DNA (Fermentas) - optional
1x Tris buffer with ethidium bromide (0.05 µg/ml)
QiaQuick gel extraction kit (you can just get the normal PCR purification kit, and buy buffer QG separately)

The genomic DNA must be digested with a restriction enzyme so it can be ligated to the linkers in the subsequent step. Use the enzyme MboI (available from many molecular companies, but we recommend Fermentas) to cut the DNA as follows: use 35-50 µg of genomic DNA in solution, put this in an Eppendorf tube with 2-3 µl (10-15 units) of enzyme, 10 µl of l0x buffer and add ddH_2O make the total reaction volume 100 µl. Incubate at 37°C in a PCR machine (alternately in a waterbath) for 2 hours. While this is incubating, pour two 0.8% agarose gels and allow the gel to solidify. After the two hour period take 5 µl aliquot of the digest and run it on the first gel together with the molecular marker and Lambda DNA. Electrophorese for

2 The MboI recognition site is ^GATC, and MboI works well for animals, and vertebrates in general. For plants it might be better to restrict with another four cutter such as MseI that has a T^TAA recognition site, and TA overhang (due to different base pair autocorrelations). The restriction conditions and linkers for ligation must be modified appropriately.

about 30 minutes at 80 volts, and check if the digestion has been complete. If the digestion is not complete, continue at 37°C and if necessary add more enzyme. Otherwise load all remaining digest onto the gel. Electrophorese for about 1 hour at 60 volts or lower to assure accurate separation.

On the transilluminator (long wavelength!), using gloves, a sterile scalpel, sterile forceps and working behind UV protective screening, cut from the gel the fraction of the electrophoresed digest that corresponds to 500-1000 base pair range of the marker. Make sure to trim as much of the gel without DNA as possible. Extract the DNA from the gel using Qiagen gel extraction kit. You may have to use more than one column since the binding capacity of the individual columns is only 10 µg; to be on the safe size, we would use two columns. Elute in small volumes, you can always dilute your fraction later. Alternately you can also add about 150 µl of sterilized Tris, melt the gel pieces in a 60°C water bath, and proceed with a normal Phenol/Chloroform extraction. If desired determine concentration of the size fraction using a spectrophotometer.

Linker preparation

Order two oligonucleotides (100 nmol setup or higher)

Er1Bh1GATCSticky	5'-GATCGGCAGGATCCACTGAATTCGC-3'
Er1Bh1Blunt	5'-GCGAATTCAGTGGATCCTGCC-3'

Make standard 100 µM stock solutions of your primers, and anneal the two primers together creating a linker DNA with a GATC overhang that complements the overhang of the digested genomic DNA. To anneal your two oligonucleotides, add 10 µl of each oligo to a 200 µl PCR tube (1 mmol each), add 0.8 µl of 5 M NaCl and then add 79.2 µl of TE, pH 8.0. Using the following PCR protocol anneal the two fragments together:

1. 95°C for 3 min.
2. 65°C for 2 min.
3. 45°C for 2 min.
4. 25°C for 1 min.
5. 4°C hold for ever

This 100 µl of annealed fragments (now referred to as linker) should provide you with enough linker for 10 size fraction-linker ligations.

Linker ligation

Materials needed:
Linker DNA (above)
5 M NaCl
TE pH 8.0
T4 DNA ligase with l0x ligase buffer (Fermentas)
3 M sodium acetate (pH 4.6)

100% ethanol
70% ethanol

or

QiaQuick PCR purification kit

To ligate the linker to your size fraction, combine the following in a PCR tube:
2-3 µg of size fraction (if you have less, use it all); whatever volume
10 µl linker (you should have about 1000:1 excess of linker to digested DNA)
10 µl l0x ligase buffer
10 units T4 DNA ligase
sufficient ddH_2O to bring up the volume to 100 µl

Using your PCR machine, ligate at 16°C overnight. Next day, clean the ligation to remove small fragments that are linker to linker ligations. You can either do it by adding 1/10 th volume of 3 M sodium acetate and two volumes of 100% ethanol. Put the tube in the -20°C freezer for about 1 hour, and then centrifuge the tube at full speed for 30 minutes, pipette off the ethanol, taking care not to remove that whitish or translucent pellet at the bottom of the tube, and wash with 200 µl of 70% ethanol. Or you may clean up your ligation using a QiaQuick column, however, this is not particularly efficient in removing small fragments above 40 bp unless guanidine hydrochloride is added during the extraction. Resuspend in 150 µl of TE or ddH_2O

<u>Size fraction-linker ligation test PCR</u>
Linker ligation
Taq polymerase (5 units/µl) and l0x polymerase buffer with $MgCl_2$ (Promega)
10 mM dNTPs (20 µl of each dNTP, 10 µl of TrisHCl and 910 µl ddH_2O)
2 µM Er1Bh1Blunt
Agarose (Fisher Low EEO)
1x Tris buffer with ethidium bromide (0.05 µg/ml)

Set up two PCR reactions with 14 µl ddH_2O, 2.0 µl 10mM dNTPs, 2.5 µl 10x PCR Buffer (includes 15 mM $MgCl_2$), 5 µl of the 2 µM Er1Bh1Blunt primer and 0.5 µl of Taq each. Mix and add 24 µl of master mix to each PCR tube, then add 1 µl of the linker ligation product. Using the following PCR protocol anneal the two fragments together:

1. 72°C for 5 min. (fill in 5' ends)
2. 94°C for 15 sec. (denature)
3. 55°C for 35 sec. (anneal)
4. 72°C for 90 sec. (extend
5. 4°C for ever

Repeat the three parameters 2-4 35 times. This cycle does not start with a 94°C step, because the initial 72°C step is required to fill in the nick between each linker and size fraction fragment left by the ligation step. While the PCR is running, pour a 1.2% agarose gel. After

PCR is completed, electrophorese both the positive and negative control PCRs against a standard size marker on the 1.2% gel for 45 minutes at 80 volts. Visualize on a transilluminator. The negative control should, of course, appear empty, while the positive lane should contain a smear of DNA that is most intense at the 500-1000 bp range. Do not be concerned if your smear ranges into very high molecular weight ranges, or even appears to start at very small sizes. The large pieces are concatamers of PCR fragments, while the small fragments were either trapped in the smear of fragments you isolated after the genomic digest, or you cut some small pieces out by accident. In any case, it does not matter at all as long as you see that smear.

<u>PCR enrichment of the size fraction-linker ligation</u>
Linker ligation
Taq polymerase (5 units/µl) and l0x polymerase buffer with $MgCl_2$ (Promega)
10 mM dNTPs (20 µl of each dNTP, 10 µl of TrisHCl and 910 µl ddH_2O)
2 µM Er1Bh1Blunt
Agarose (Fisher Low EEO)
1x Tris buffer with ethidium bromide (0.05 µg/ml)
QiaQuick PCR purification kit

Set up 16-24 PCR reactions with 28 µl ddH_2O, 4.0 µl 10mM dNTPs, 5 µl 10x PCR Buffer (includes 15 mM $MgCl_2$), 10 µl of the 2 µM Er1Bh1Blunt primer and 0.5 µl of Taq each. Mix and add 48 µl of master mix to each PCR tube, then add 1 µl of the linker ligation product. Using the following PCR protocol anneal the two fragments together:

1. 72°C for 5 min. (fill in 5' ends)
2. 94°C for 15 sec. (denature)
3. 55°C for 35 sec. (anneal)
4. 72°C for 90 sec. (extend)
5. 4°C for ever

Repeat the three parameters 2-4 12 times. This cycle does not start with a 94°C step, because the initial 72°C step is required to fill in the nick between each linker and size fraction fragment left by the ligation step. The reason for doing the 16-24 separate PCR reaction at only 12 cycles is to maintain the complexity of the linker ligation mixture. This is important, otherwise you will be sequencing a lot of identical clones in the end. Pool your PCR products and clean on QiaQuick columns. You will have to load each column several times, and also use 3-4 columns in order to not overload the columns. Combine the elutions to yield approximately 150 µl of cleaned PCR product. This is now the pool of fragments that you will fish microsatellites out from.

The next two steps should be started the previous day, and completed, along with the step after that, while the above PCR is running.

<u>Biotinilation of microsatellite probe</u>

Order oligonucleotides (100 nmol setup or higher)
MicCA 5'-CACACACACACACACACACA-3'
MicCT 5'-CTCTCTCTCTCTCTCTCTCT-3'
and resuspend them to a 40 mM concentration
Biotin 14-d-ATP (Invitrogen) (comes with 5x buffer)
Terminal Deoxynucleotidyl transferase with 5x buffer (Fermentas)
3 M sodium acetate (pH 4.6)
100% ethanol
70% ethanol

Attach biotin to the microsatellite probe by adding to an Eppendorf tube the following:
10 µl 40 µM oligonucleotide
1 µl of Biotin 14-d-ATP Biotin
8 µl 5x Terminal transferase buffer
0.5 µl terminal transferase
15.5 µl ddH2O

Incubate the reaction in at 37°C for exactly 25 minutes. At 25 minutes, remove the tube from the water bath, add 4 µl of 3 M sodium acetate, 100 µl or 100% ethanol, and put in the -20°C freezer overnight. Note: Buffers come with $CoCl_2$. Cobalt chloride is a toxic chemical, so should be treated as such. Wear gloves.

The next day, while the PCR from the previous step is running, centrifuge the biotinilation reaction at 13,000 rpm for 30 minutes. Remove the ethanol, wash the pellet (almost, if not impossible to see) with 70% ethanol, spin for another five minutes, remove the ethanol, dry for a few minutes in a Speed Vac, and resuspend in 100 µl ddH_2O.

<u>Preparing the Dynabeads and forming the biotin/streptavidin complex</u>

Dynabeads M-280 Streptavidin (Dynal Biotech www.dynal.net – now acquired by Invitrogen)
Phosphate-buffered saline (PBS), pH 7.4 with 0.1% BSA.
B&W Buffer (see Dynal info sheet included with Dynabeads)
Strong magnet for concentrating beads (one of the MPC ones from Dynal, or jerry-rig your own)
1.5 ml screw top Eppendorf tubes with lids, autoclave sterilized

Dynabeads are metal beads with an avidin protein attached to the surface. This is the avidin that you will complex your biotinilated probe to. Dynabeads come with a 0.02% NaN_3 preservative in the storage solution that must be removed before attaching the probe. Once the probe has been attached, the bead/probe complex is stable in the fridge for months. We have successfully used complexed beads a year after attaching the probe.

Take your tube of Dynabeads and pipette 200 µl or beads into a screw top Eppendorf after first resuspending the beads in the storage tube by pipetting the storage buffer up and down. Screw the cap on the tube. Use your magnet to get the beads out of solution against the side of

the tube. Keeping the tube in your magnet concentrator, pipette off the storage solution and add 200 µl of the PBS/BSA solution. Finger vortex this vigorously so that the beads go back into solution and are no longer stuck to the side of the tube. Put your tube back into the magnet concentrator again and let the beads come out or solution. Pipette off the initial wash solution, and repeat the wash with PBS/BSA two more times. After the second wash, remove the solution again in the magnet concentrator and replace with 200 µl of the B&W solution. Finger vortex again, separate the beads in solution into two screw top Eppenedorf tubes (l00 µl per tube) and store in the fridge until you are ready to complex your probe to the beads in one tube. Note: do NOT freeze your beads, ether when they arrive, when they are washed or after the probe has been complexed.

Once you have your biotinilated probe prepared and in 100 µl of ddH_2O, add the entire probe solution to one of the tubes of beads. Make sure the tube of beads is at room temperature first. Leave the tube standing at room temperature for one hour, then put the tube of complexed beads in the fridge until you are ready to fish out your microsatellites.

Hybridizing probe to the microsatellites: The magic step

Bead probe complex
B&W Buffer
Autoclave tape
1 15 ml tube of 5x SSC, 0.1% SDS
1 15 ml tube of l0x SSC, 0.2% SDS
2 15 ml tubes of 2x SSC, 0.1% SDS
1 15 ml tube with 990 µl 1x TE and 10 µl 5 M NaCl
TE

This is the point where you isolate your potential microsatellites from your enriched pool of subgenomic DNA. If this step is done improperly, you can either lose your fragment-linker ligation or end up fishing out a lot of non-microsatellite DNA, reducing the efficiency of your subsequent library. If you do things correctly, you can end up with as many as 70% of all your subsequent clones containing microsatellite regions. There are no tricks to this step, so if you follow the protocol, you will have success.

First, preheat your hybridization oven to 55°C. Put the 15 ml Corning tube containing the l0x SSC, 0.2% SDS and one of the 2x SSC, 0.1% tubes in the oven for prewarming. If your 5x SSC, 0.1% SDS solution has white precipitate in it, heat this tube as well, but allow it to cool to close to room temperature before using. Aliquot 150 µl of your cleaned PCR pool of fragments into a 200 µl PCR tube; cycle the tube in your PCR machine for 5 minutes at 95°C to thoroughly denature the DNA, and snap-cool on ice. Take your tube of bead probe complex (we will simply call it beads from now on) and wash the beads twice, using the magnet concentrator, with 200 µl of the B&W solution. Wash once more with 200 µl of the near-room temp 5x SSC, 0.1% SDS. Remove the 5x SSC solution and add 150 µl of the prewarmed l0x SSC, 0.2% SDS solution. Keep the tube at 55°C as much as possible now. Add the snap-cooled fragments to this

tube, screw the top onto your Eppendorf very tightly, and tape the tube to the rotating part of the hybridization oven. Make sure you tape the tube with autoclave tape, as typical lab tape will lose its stickiness when exposed to 55°C for a prolonged period. Turn on the rotor and incubate your tube for 3-4 hours at 55°C. Periodically check your tube in the oven. Over the course of the hybridization, the beads can become stuck to the side of the tube and therefore not function in hybridizing microsatellite fragments. If you see beads accumulating on the side of the tube, stop the rotor, untape the tube, finger vortex vigorously until the beads come lose, retape the tube and turn on the rotor again. These periodic breaks during the hybridization do not seem to affect hybridization efficiency.

After the hybridization period, untape the tube and wash the hybridized beads twice at room temperature, using 200 µl of the room temperature 2x SSC, 0.1% SDS. For each wash rotate the tube by hand for five minutes before pipetting off the wash solution. Note: save each wash solution and the original supernatant from the hybridization in case you were unsuccessful with the hybridization step. Although we have never had to do this, if you have not succeeded in hybridizing any microsatellite DNA, it is feasible to reprecipitate your DNA from any of the solutions and retry the hybridization using the same DNA. After the two five minute washes, wash the beads once with 200 µl of the prewarmed (55°C) 2x SSC, 0.1 % SDS. You need to wash for 10 minutes for this step, so retape the tube onto the rotor and use the oven to invert your tube for ten minutes. Make sure your lid is screwed in tightly! Again, keep the supernatant. After pipetting off the 2x SSC, 0.1 % SDS, wash the beads once with 200 µl of the room-temperature TE/NaCl solution, pipette off the solution using the magnet, and resuspend the beads in 200 µl of TE. At this point, if all has worked well, you have found your microsatellites, now they just need to be cloned and sequenced!

PCR test of the hybridized beads

Linker ligation
Taq polymerase (5 units/µl) and l0x polymerase buffer with $MgCl_2$ (Promega)
10 mM dNTPs (20 µl of each dNTP, 10 µl of TrisHCl and 910 µl ddH_2O)
2 µM Er1Bh1Blunt
Agarose (Fisher Low EEO)
1x Tris buffer with ethidium bromide (0.05 µg/ml)

Set up two PCR reactions with 14 µl ddH_2O, 2.0 µl 10mM dNTPs, 2.5 µl 10x PCR Buffer (includes 15 mM $MgCl_2$), 5 µl of the 2 µM Er1Bh1Blunt primer and 0.5 µl of Taq each. Mix and add 24 µl of master mix to each PCR tube, then add 1 µl of the enriched linker-ligation product. Using the following PCR protocol, anneal the two fragments together:

1. 94°C for 15 sec. (denature)
2. 55°C for 35 sec. (anneal)
3. 72°C for 90 sec. (extend)
4. 4°C for ever.

Repeat the three parameters 1-3 35 times. While the PCR is running, pour a 1.2% agarose gel. After PCR is completed, electrophorese both the positive and negative control PCRs against a standard size marker on the 1.2% gel for 45 minutes at 80 volts. Visualize on a transilluminator. The negative control should, of course, appear empty, while the positive lane should contain a smear of DNA that is most intense at the 500-1000 bp range.

PCR amplification of hybridized microsatellites and ligation
pCR4 or pCRII TOPO TA cloning kit (Invitrogen)[3]
Taq polymerase (5 units/µl) and l0x polymerase buffer with $MgCl_2$ (Promega)
10 mM dNTPs (20 µl of each dNTP, 10 µl of TrisHCl and 910 µl ddH_2O)
2 µM Er1Bh1Blunt
Agarose (Fisher Low EEO)
1x Tris buffer with ethidium bromide (0.05 µg/ml)
QiaQuick PCR purification kit

Set up 4 PCR reactions with 14 µl ddH_2O, 2.0 µl 10mM dNTPs, 2.5 µl 10x PCR Buffer (includes 15 mM $MgCl_2$), 5 µl of the 2 µM Er1Bh1Blunt primer and 0.5 µl of Taq each. Mix and add 24 µl of master mix to each PCR tube, then add 1 µl of the linker-ligation product. Using the following PCR protocol anneal the two fragments together:

1. 94°C for 15 sec. (denature)
2. 55°C for 35 sec. (anneal)
3. 72°C for 90 sec. (extend)
4. 72°C for 30 min (final extend)
5. 4°C for ever

Repeat the three parameters 1-3 15 times to maintain complexity of the linker-ligation mixture. This is important, otherwise you will be sequencing a lot of identical clones in the end. Pool your PCR products and clean on a QiaQuick columns. Elute with minimal volumes to get large concentrations of DNA product, you should have about 200 ng/µl. Do this immediately after the PCR reactions, and be ready to clone. Make sure that you amplify using Taq polymerase, and not some other polymerase variant (e.g., Deep Vent polymerase, Pfu, Pwo). This is important as Taq polymerase adds single A overhangs to the 3' end of PCR products as an artifact of PCR. These overhangs are lost rapidly after the reaction is completed. The TA cloning kit includes a cloning vector (plasmid) that has been blunt cut and then a 3' single T overhang added. This kit, therefore, is especially designed to ligate fresh PCR fragments.

As you might have guessed, you need 200 ng of PCR DNA for your ligation. Invitrogen suggests using 10 ng of DNA for ligation in their TA vector, but we have found that 200 ng

3 The protocol described here is for the TOPO TA cloning kit sold by Invitrogen. It is highly efficient and easy, but as you have noticed, the linkers are designed with EcoRI and BamHI restriction sites, so you can also clone them into for example BlueScript vector after EcoRI or BamHI digestion of the vector and the linker-ligation product. You will need to do blue/white selection with IPTG/X-Gal when doing the BlueScript cloning. Also lately we have been using the equally efficient pJET cloning kit from Fermentas.

generally results in the most efficient ligation in this case. To ligate your fresh PCR into the TA vector, follow the directions provided with the TA kit, with the following modifications with the exceptions of using more DNA, and you can easily use only 2 the recommended volume of the vector. Ligate for the maximal 30 minute time duration, and you are ready to clone. Freeze the rest at -20°C for later use.

The TOPO TA kit can be ordered with One Shot or some kind of TOPO competent cells from Invitrogen. As an alternative, you can make your own chemically competent cells using some standard methodology. Make sure you pour your media plates for use during the transformation you will be doing the next day. How to make LB media, pour plates and include antibiotic, inducer and X-Gal in plates can be found in any standard text on microbiological or molecular techniques, e.g. (Sambrook et al. 1989).

Transformation of the Library

linker-ligation library
SOC or LB media (provided with Invitrogen competent cells), prewarmed to 37°C
LB plates with Ampicillin, X-Gal and IPTG
Sterile spreader
Shaking incubator (37°C)
Water bath or heating block (42°C)
Ice
Nonshaking incubator (37°C)

Depending on the competent cell type you use, follow the TA cloning kit protocol for chemical transformation. In general put in 2 µl of the transformation per one tube of competent cells (50 µl). Let the cells thaw on ice, mix very gently and allow the cells to take up the linker-ligation library for 30 minutes. Heat shock the cells for 50 sec at 42°C. Add 250 ml of SOC or LB to the cells, and incubate with gently (200 rpm) for 1 hour in a shaking incubator. During this stage be very careful about contamination since there is no ampicillin in the medium.

A day ahead prepare LB/amp plates (if you want you can also add X-Gal and IPTG, but in the case of the TOPO TA cloning there is no need for blue/white selection). Plate about 50 µl of the transformed cells per place (more or less as based on your own experience). Incubate plates at 37°C for at least 8 hours, or best overnight. You should get hundreds of colonies. If they are too dense, hard to pick and overlap, decrease the volume of cells plated.

Picking white colonies for replicate plates

Cut off toothpicks, or sterile pipette tips for picking colonies
96 well cell culture plates
Liquid LB/amp media
Glycerol
Ampicillin

Sterile fume hood
Replica plater (hedgehog)

You probably have plates jammed with white colonies, so doing a standard colony lift and screening this membrane for microsatellites is an exercise in futility. You will end up with a membrane covered in positive signal, making individual positive clones impossible to pick reliably. Instead, it is better to pick a selection of individual colonies and grow them in replica plates that you will screen for positive clones. For each master plate (96 wells, therefore, 96 white colonies, we generally pick two master plates for each initial screen), make up 15 ml of glycerol LB media (15 ml liquid LB media and 150 µl ampicillin stock solution) and aliquot 150 µl into each well of a 96-well cell culture plate (work in a sterile hood). Pick individual colonies, put into well with the medium making sure there is no cross-contamination. Grow in a 37°C incubator for 4 or more hours (you can do it overnight, but try not to overgrow the cells).[4]

After the growth period, you can dot blot your bacteria to see if they contain positive clones. We find this a waste of time since 50+% of the clones have microsatellite repeats. We just proceed directly with PCR and sequencing of all the clones.

PCR of clones

Order oligonucleotides (100 nmol setup or higher)

M13 Forward (-20)	5'-GTAAAACGACGGCCAG-3'
M13 Reverse	5'-CAGGAAACAGCTATGAC-3'
T7 Promoter	5'-TAATACGACTCACTATAGGG-3'
T3 Promoter	5'-ATTAACCCTCACTAAAGGGA-3'
or	
SP6 Promoter	5'-ATTTAGGTGACACTATAG-3'

Taq polymerase (5 units/µl) and l0x polymerase buffer with $MgCl_2$ (Promega)
10 mM dNTPs (20 µl of each dNTP, 10 µl of TrisHCl and 910 µl ddH_2O)
2 µM of each of the M13 forward and reverse primers
Agarose (Fisher Low EEO)
1x Tris buffer with ethidium bromide (0.05 µg/ml)
QiaQuick PCR purification kit

Set up a PCR reaction for each well (96 well plate preferably) with 14 µl ddH_2O, 2.0 µl 10mM dNTPs, 2.5 µl 10x PCR Buffer (includes 15 mM $MgCl_2$), 2.5 µl each of the 2 µM M13 forward and M13 reverse primer and 0.5 µl of Taq each. Mix and add 24 µl of master mix to

4 If you want, set up a replicate plate with 20% glycerol solution in the LB/amp (make up 15 ml of glycerol LB media -12 ml liquid LB media, 3 ml glycerol and 150 µl ampicillin solution - and aliquot 150 µl into each well of a 96-well cell culture plate (work in a sterile hood). Use a hedgehog or just a multichannel pipette to replicate the plate. Once each well has been inoculated, incubate the 96-well plate for three hours at 37°C. This initial master plate will eventually be stored at -80°C and will act as the reference plate in case any disasters happen with the plates you are using.

each PCR tube, then add 2-3 µl of the LB medium with bacterial cell growth. Using the following PCR protocol:

1. 94°C for 5 min. (kills *E. coli* and breaks up cells releasing plasmids)
2. 94°C for 15 sec. (denature)
3. 55°C for 35 sec. (anneal)
4. 72°C for 90 sec. (extend)
5. 72°C for 7 min. (final extend)
6. 4°C for ever

Repeat the three parameters 2-4 35 times. We prefer to directly PCR the LB medium with bacteria rather than doing a miniprep, since it is faster, and we think it even works better. After the PCR is done, clean your PCR products a QiaQuick columns. You should get a lot of DNA product, so elute with 80 ml of the buffer EB (TE buffer).

Clone sequencing

Purified PCR product
2 µM of each of the T7 and T3 or SP6 primers[5]
ABI BigDye
BigDye 5x replacement buffer

Make a master mix by adding 3.0 µl ddH_2O, 2.0 µl 2 µM primer and 0.5 µl of BigDye and 2.5 µl of BigDye 5x replacement buffer[6]. Mix and add 8 µl of master mix to each reaction, and add 2 µl purified DNA.

1. 96°C for 10 sec. (denature)
2. 50°C for 05 sec. (anneal)
3. 60°C for 240 sec. (extend)

Repeat the first three parameters 35 times (the standard protocol says 25 times, but since we are using 0.5 µl of the BigDye, we have found it to give better resolution for longer sequencing reactions with greater number of extensions). If you are not getting good results, use more BigDye. However, beware that your sequencing reaction will almost always drop of after sequencing through the microsatellite, hence you will need to sequence in both direction. On that note, it may be best to first sequence just with just one of the internal primers (e.g. T7) and then sequence only those positive clones with the other primer. Also note that we have amplified with M13 forward and reverse primers, and then sequenced with T7 and T3 or SP6

5 Use T7 and T3 as internal sequencing primers for the pCR4 TOPO TA cloning kit, and T7 and SP6 as internal sequencing primers for the pCRII TOPO TA cloning kit.
6 ABI now provides the 5x replacement buffer with their sequencing kits. The 5x replacement buffer is 400 mM Tris-HCl pH 9.0 and 10 mM $MgCl_2$. The BigDye sequencing mix itself has a 2.5x 'buffer' – you are supposed to use 8 µl in a 20 µl reaction. So theoretically you should use just under 2 µl of the 5x replacement buffer in a 10 µl reaction, but 2.5 µl appears to produce more robust results or at least does not hurt.

primers which are internal to M13 forward and reverse. This gives consistently better sequencing results. Thus it is important that you get the correct TOPO TA cloning kit where the vector has four priming site and not three or less (such as pCR2.1 TOPO TA cloning kit).

Primer design and population screening

Once you have your clones sequenced, you will need to design appropriate primers to amplify that repeat in the organism of interest. As a general rule, try to pick perfect repeats, and those larger than 10 repeat units, as these are almost invariably polymorphic. Design your primers well, making sure they have relatively high annealing temperatures (around 60°C), and that at least one of the 5' ends of the primers are likely to have the A overhang added by Taq polymerase (Brownstein et al. 1996) – this will be important once you make labeled primers and do actual population scoring. Screen you population, pick out good primers, and label them, but consider the cost saving technique described by Schuelke (2000).

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Solutions

TBE (10X)
Tris base – 216 g
Boric acid – 110 g
EDTA 0.5 M (pH 8.0) – 80 ml
Dissolve in sufficient amount of distilled H_2O to give 2 L, and filter

TE (1X)
Tris 10 mM
EDTA 0.5 – 0.1 mM

TE/NaCl
TE 1 M = 990 µl
NaCl 5 M = 10 µl

B&W Buffer (Binding & Washing Buffer (2X))
Tris-HCl 10 mM (pH 7.5)
EDTA 1 mM
NaCl 2 M

PBS (g for 500 ml volume)
137 mM NaCl (4g)
2.7 mM KCl (0.1g)
10 mM Na_2HPO_4 (0.72g)
2 mM KH_2PO_4 (0.12g)

PBS/BSA (Saline phosphate buffer pH 7.4)
125 ml PBS
1.06 g NaCl
1 ml 0.5 M EDTA
Add milli-Q H_2O to 300 ml
0.3 mg BSA (0.1% w/v)

SSC (20X) (Saline Sodium Citrate)
175.35 g NaCl
88.20 g NaCitrate·$2H_2O$
Dissolve in sufficient amount of milli-Q H_2O to give 1 L, and filter

SDS 10% (keep at room temperature)
1 g SDS
10 ml milli-Q H_2O

SSC 5X /SDS 0.1%

2.5 ml SSC (20X)
100 µl SDS 10%
Add sufficient amount of milli-Q H_2O to give 10 ml

SSC 10X /SDS 0.2%
5 ml SSC (20X)
200 µl SDS 10%
Add sufficient amount of milli-Q H_2O to give 10 ml

SSC 2X/ SDS 0.1%
1 ml SSC 20X
100 µl SDS 10%
Add sufficient amount of milli-Q H_2O to give 10 ml

LB agar (Luria-Bertani agar)
10 g NaCl
10 g Bacto tryptone
5 g Bacto yeast extract
15 g agar
Add sufficient amount of distilled H_2O to give 1 L, and autoclave

LB broth (Luria-Bertani broth)
10 g NaCl
10 g Bacto tryptone
5 g Bacto yeast extract
Add sufficient amount of distilled H_2O to give 1 L, and autoclave

SOC Broth (1 liter)
20 g Bacto tryptone
5 g Bacto yeast extract
0.6 g NaCl
0.5 g KCl
10 ml of 2 M $MgCl_2$ + $MgSO_4$ (see below)
20 ml of 1 M Glucose (see below)
Dissolve tryptone, yeast extract, sodium chloride, and potassium chloride in a final volume of 970 ml distilled H_2O
Sterilize by autoclaving
After autoclaving, allow the solution to cool to 60°C, and add 20 ml of a sterile 1 M glucose stock (see below) to make the media 20 mM with respect to glucose
Just prior to using, add 10 ml of magnesium stock (see below) to the SOC broth to make the media 20 mM with respect to magnesium.

2 M Mg_2+ stock (100 ml)
20.3 g $MgCl_2$

24.7 g $MgSO_4$
Dissolve reagents in a final volume of 100 ml distilled H_2O. Sterilize by filtration through a 0.45 µm disposable filter. The resulting solution is 2 M with respect to Mg_2+.

1 M Glucose (100 ml)
18.0 g glucose
100.0 ml dH_2O
Dissolve glucose in 90 ml distilled H_2O. Bring to final volume of 100 ml. Sterilize by filtration through a 0.22 µm disposable filter.

Ampicilin
Stock solution: 10 mg/ml
Working solution (concentration in LB): 100 µg/ml

Tris-Calcium (1 M $CaCl_2$ + 1 M Tris-HCl pH 7.5)
1 ml 1 M Tris-Calcium
7 ml 1 M $CaCl_2$
Add sufficient amount of distilled H_2O to give 100 ml, dissolve by boiling in microwave