

Appendix I*

Making a microsatellite DNA library by hybridisation enrichment

Acknowledgements – This protocol is derived from one described by Tania King, which came originally from that of Armour *et al.* 1994 (*Human Molecular Genetics* **3**:599-605). I Thank Niccy Aitken, Leon Huynen, Hilary Millar, Pete Ritchie, Gwilym Haynes, Mathew Chan and Jenny Hay for assistance.

“Let time work for you”

Peter Ritchie, Palmerston North, 2000

Most of the reagents required for library construction are listed on page 141.

1. Start by making probes

Choose repeat motifs.

I would suggest tetranucleotides because when it comes to genotyping, your job will be a lot easier. Most people opt for dinucleotides because they are supposed to be more common. I had no trouble finding enough tetranucleotides for my study (10 loci). It is little extra work to try multiple motifs because they can be run simultaneously. I'd suggest trying a number of different ones, maybe both di- and tetranucleotide.

Order oligonucleotides.

I ordered (AAAG)₇, and its match (CTTT)₇ from Sigma-Genosys, also (GATA)₇/(TATC)₇. They cost about \$50 a pair.

DNA, particularly single stranded DNA like these oligonucleotides should be stored as high pH solutions –not water! Best is TE pH 8.0.

The Oligos from Sigma came dehydrated. I make up to a standard concentration of 1 nmol/μl. This is the stock solution. I then made a working solution of 10 pmol/μl (=10uM) as a working stock. So that's a 1/100 dilution from the stock –I used water to make the dilution. Oligos get stored in the –20°C freezer.

Make lig-concatemers

i.e. Join the oligonucleotides together to make longer oligonucleotides.

1. *Phosphorylation* (add a phosphate group to the end of the oligo to facilitate ligation to others) and *annealing* (join the strands to their complement).

For each pair of oligos combine the following in a tube that will fit a PCR machine (e.g. 0.5ml):

2ug of each oligo

Ligase buffer (to 1x buffer overall)

* This appendix is not intended for examination, but is included to provide a complete record of laboratory protocols.

MilliQ water to 50 μ l

Heat to 70°C for 5 minutes –use PCR machine.

Chill on ice (until chilled –a couple of minutes).

Add 3-5 μ l T4 PNK. –this adds a phosphate group to the 3' end so that the oligos will ligate to one another (there is a free -OH group on the 5' end).

Incubate at 37°C for 45 minutes

Heat denature at 65°C for 20 minutes

2. *Now ligate them together...*

The 50 μ l annealed oligos from before

Ligase buffer (to 1x buffer overall –including that already in the mix)

T4 DNA ligase 1 μ l.

MilliQ water to 65 μ l total

Incubate overnight at 16°C. (eg. put a waterbath in a 4 degree room)

3. *Isolate these 'lig-concatemers'* by size fractionating them from a *ca.* 1.2% agarose gel.

Use one large well per probe for the 65 μ l ligation mix (about 3 standard sized wells combined with tape) and gently fill. Run a 100bp ladder down each side of the main well. Don't run the gel too long –you want to minimise the amount of gel you have to cut out –just long enough so that you can see >200bp. Low voltage is good –about 100v.

Good practice: Put saran wrap on the UV transilluminator so you don't contaminate your DNA. With the UV on, quickly make marks where the DNA >200 bp is. Turn the UV off and cut out DNA >200bp with a sterile blade. Recover the DNA from the agarose using a kit (eg. Roche Highpure).

4. *Make giant probes* by 'primer-free' PCR. Do about 5 replicates for each probe (i.e. AAAG/TTTC and GATA/TATC)

1 x Buffer

6.7mM (!) MgCl₂

0.8mM dNTPs

2u Taq

1 μ l lig-concatemer template

to a total volume of 25 μ l.

For an I-cycler try a PCR cycle of: 95°C for 2 mins, [55°C for 20 secs, 72°C for 30 secs, 95°C for 30 secs] x 30, 72°C for 5 mins. Tanya used 55°C for 1 min, 72°C for 2 min, 95°C for 1 min on the old Hybaid Omni-E machine. A greater yield may be achieved with lower annealing temperatures.

Run out 2 μ l or so of each. You should see a big smear out of the wells to ~100bp.

Pool all the replicates and ethanol precipitate. Resuspend on ~ 50 μ l milliQ water. Quantify. I got ~ 2 μ g/ μ l. Dilute to 1 μ g/ μ l.

Put them in the fridge.

Next,

2. Prepare your animal DNA

Extract DNA from about 5 individuals (both sexes if you don't know the heterogametic sex, or just use the heterogametic sex – if lucky you may find a sex marker). Digest each separately with NdeII (=MboI=Sau3A).

5µl genomic DNA

1 x buffer

1µl enzyme

0.5 µl RNase

milliQ water to a total vol of 15 µl.

Digest overnight at 37 °C. An extra 0.5 µl of enzyme can be added later in the digestion.

Quantify the DNA from each digestion.

Combine these samples (equally) to get a total of 5-10ug DNA. Run the DNA out on a ~1% agarose gel (same procedure as above) and cut out DNA between 300 and 800 bp, again minimising the amount of gel to cut out. Tanya cut out 300-600bp, but I found that my microsats often didn't have much flanking sequence on one side –that may have been because they were tetras and very long. Recover the DNA from the agarose. Quantify it. I usually got between 10-40ng/µl.

Make 'SAU linkers'

Order the two halves and make up working concentrations with TE (I made it 1 nmol/µl)

SAULA (5' –GCGGTACCCGGGAAGCTTGG-3')

SAULB (5' –GATCCCAAGCTTCCCGGGTACCGC-3') (GATC is the overhang).

You want 5ug of SAULB and an equimolar amount of SAULA. Based on a 1nmol/µl working stock of SAULB, you will need 0.689 µl to make 5ug (a 1nmol/µl SAULB = 7.26 ug/µl). That's 0.689 nmoles and you'll also need 0.689 µl of SAULA.

To make the linker:

0.689 µl SAULA

0.689 µl SAULB

1.0 µl React 1 buffer (Gibco)

7.62 µl Q (a total volume of 10µl).

Make a 5 or 10 x master mix so you have replicates and the pipetting is more accurate.

Cycle through the following:

95°C - 40 secs

65°C – 15 min

60°C –15 min

55°C –15 min

50°C – 15 min

45°C – 15 min

40°C – 15 min

hold at room temperature.

Quantify it. I got ~1.12ug/μl (you put in about 10ug in 10μl).

Ligate the linkers onto the DNA

Ligate *ca.* 200ng DNA to 2.75 ug linker (a 1:250 molar ratio).

2.75ug SAU linker

200ng DNA

1 x ligase buffer*

1.0 μl T4 ligase

milliQ water to a total volume of 40 μl.

Incubate overnight @ 16°C in a waterbath.

* Ligase buffer contains ATP, which is sensitive to freeze-thaw. If you suspect it is degraded add 2μl of 10mM ATP to the ligation.

Put the ligation mix through a PCR purification kit to get rid of the unligated linkers (I used Highpure by Roche). Quantify. I got between 12.5 and 185ng/μl.

Pre-enrichment PCR (Tanya suggested that if you use a lot more DNA in the previous steps that this step can be left out. I did a pre-enrichment PCR, but fewer PCR steps could be better because PCR errors such as recombination may be less likely).

1x Buffer

3mM MgCl₂

0.8mM dNTPs

0.5um SAULA

10ng DNA template

1 U *Taq*

MilliQ water to 25 μl.

For the I-cycler I cycled through: 95°C –5min, [58°C – 30 sec, 70°C – 20 sec, 95°C – 30sec] x 30, 70°C - 4 min. On the Hybaid try: : 95°C –5min, [58°C –1min, 70°C – 2min, 95°C – 1min] x 30, 70°C - 4 min. In hindsight, I would try a gradient of annealing temperatures and probably use a lower annealing temperature.

Run out ~5μl from each reaction on a gel. You should see a smear between 300 and 800bp. Pool the reactions and ethanol precipitate them or put through a PCR purification column, resuspending on ~20μl. This is the size-selected DNA template that you will enrich from. Quantify. I got 420-792 ng/μl. You may need to pool multiple pre-enrichment runs.

3. Enrichment

Make some micro-membranes

Procure a small piece of membrane (eg. Gelman)

Being careful to avoid contamination cut out 30 or so squares about 2.5mm x 2.5mm.

Store them in a 1.5ml tube.

Denature the probes

1ug probe

1µl 2M NaOH
1µl 20mM EDTA
milliQ water to 9µl

Set up 10 replicates for each probe and incubate them at 37°C for 30 minutes.
Neutralise with 1µl 1M Tris HCL pH 4.8, incubate for 10 minutes at room temperature.

Meanwhile, get a light table and put a couple of bits of gladwrap on it -label one for each probe with a permanent marker. Lay out 10 micro-membranes on each sheet.

Dot 1-2µl probe at a time onto each micro-membrane. You don't want the probe to run off the membrane so don't do too much at a time and let them dry before the next round. This is tedious. The light table is to speed up the drying process -its optional and you could use some other warm surface. You are aiming to put 1µg of probe per micro-membrane.

Once the membranes are all dried, fold gladwrap over the top and expose to the UV transilluminator for *ca.* 50 seconds per side. This is intended to crosslink the probes and bind them to the membrane. Store these in the -20°C freezer until use.

Hybridisation

Two options: you could run the hyb with both probes + DNA in the same tube, or each probe + DNA in separate tubes. It may not make a difference unless you definitely want a specific microsatellite motif. You should also run an extra tube(s) with membranes but no DNA added as a pseudo hyb-control.

Make 1300 µL of Church and Gilbert solution + 1% BSA for each tube you will run. Use a stock C&G soln. but add the BSA fresh (i.e. 10mg BSA powder per 1mL C&G soln.).

Pre-hyb membranes in 1ml of C&G/BSA at 65°C for 3+ hours.

After a couple of hours....

Denature your DNA in the same way as the probe.

1µg DNA
1µl 2M NaOH
1µl 20mM EDTA
MilliQ water to 9µl

Incubate at 37°C for 1 hour

Neutralise with 1µl of 1M Tris HCL pH 4.8, incubate for 10 minutes at room temperature.

Draw out the pre-hyb mix with a pipette leaving the membranes. Add 200 µL fresh C&G/BSA to the denatured DNA, mix and then add the mixture to membranes.

Hyb. overnight at 65°C rotating.

Washing

Note. Originally I processed my hybs and the hyb control at the same time. I spent a long time puzzling over the source of contamination in the hyb-control. It came from

the true-hyb - so its worth being fastidious and taking your time (the DNA in the hyb is PCR product so its very easy to contaminate). Do the whole washing procedure for the hyb-control before you start on the true-hyb. Also, make sure you spin the tubes down between each step. In hindsight, there is actually not much point in having the hyb control unless there have been a lot of libraries constructed in your lab with the same SAU linkers etc. It controls for contamination with SAU linkers but not much else.

Draw out the hyb liquid. Wash 3 times (at 65°C /rotating) for 10 minutes with 600 µl pre-warmed 2 x SSC/0.1 % SDS. Rinse at room temperature with 5 x SSC (no SDS). Draw out the liquid and air dry the membranes in the tubes.

Recover DNA

Add 100µl 50mM KOH/0.01% SDS. Stand with occasional flicking at room temperature for 10 minutes. Add 100µl 50mM Tris HCL pH 7.5/0.01% SDS. Flick a bit, take out membranes.

Precipitate DNA

1µl glycogen (20mg/ml) (this acts as a carrier for the DNA)

20µl 3M NaOAc pH 5.2

500µl 100% ETOH

Chill 10 minutes, spin 30 mins, wash with 500 µl 70% ETOH, spin again, dry, resuspend on 6µl milliQ water.

Amplify the enriched DNA (x 6)

1x Buffer

3mM MgCl₂

0.8 mM dNTPs

0.5 µM SAULA

1 U Taq

1 µl BSA

1µl DNA

MilliQ water to 10µl.

For the Hybaid: 95°C - 5 min, [67°C – 1min, 72°C – 1min, 95°C – 1min] x 35, 72°C – 4min. Alter it for the I-cycler –maybe 30 secs per stage. I am not sure why Tanya used such a high annealing temperature. A less stringent temperature would be better. Perhaps use the same temperature you found to be optimal in the pre-enrichment PCR.

Pool PCRs and size fractionate 300-800bp DNA on a 1.2 % agarose gel. Recover DNA with a kit. Minimise the amount of gel to cut out by running only for a short time. You should see a smear between 300-800bp.

If you did one, also run a PCR using hyb-control - there shouldn't be a product.

Digest the DNA with NdeII to get rid of the 'SAU-linkers' and leave GATC 'sticky ends'. Incubate at 37°C for 2 hours. Deactivate enzyme by heating for 20 mins at 65°C. Put through a PCR purification kit to get rid of the linkers. This is your insert DNA. Quantify. I got 20 ng/µl. Having a good amount of DNA is v. important here –I ended up pooling the results of 2 hybs and ethanol precipitating them.

4. Cloning

Ligation of insert into a pUC18 plasmid vector cut with BamHI

Work out the appropriate quantities of DNA and plasmid to get the right molar ratio (insert:plasmid). I had success with a molar ratio of 7:1 and 10:1, but Tanya used 3:1.....

e.g.
$$\frac{\text{Ave. size insert}}{\text{Size pUC18}} \times \frac{7}{1} \longrightarrow \frac{550}{2700} \times \frac{7}{1} = 1.43$$

I used 50ng of pUC18 in the ligation, so you would need 1.43 x 50 ng of insert (= 71.5ng).

1x ligase buffer

1µl T4 DNA ligase

pUC18/BamHI

Insert DNA

MilliQ water to 10µl

Incubate overnight @ 16°C. Don't heat de-activate.

Transformation of ligated plasmids into cells

First, make agar plates –ten per transformation and let them cool fully (see appendix).

I used DH5α cells from Lifetech and pretty much followed their instructions.

Dilute the ligation mix 5-fold with TE (pH 7.5).

Put 100µl cells in a 10 ml tube, and add 1.5µl ligation mix, moving the pipette gently while dispensing.

Incubate on ice for 30 minutes.

Heat shock in a water bath @ 42°C for 45 seconds.

Place on ice for 2 minutes.

Add 900µl SOC buffer, place on shaker for 1 hour (or a bit longer) @ 225 rpm @ 37°C.

The mix should go a bit cloudy.

Spread 100µl of transformation mix evenly onto each plate. When dry, put lid on, turn upside down and allow to grow overnight at 37°C.

Colonies containing recombinant plasmids will show up white and non-recombinant blue. When the colonies are big enough to identify colour, pick them into a microtitre plate (see below). Putting the plates at 4°C can help the colour come up.

Medium for picked colonies

Microtitre plates have 96 wells and you'll need about 100µl per well (depends on the length of the hedgehog spikes).

80µl L-broth per well

20µl glycerol per well

0.1µl Ampicillin per well (@50mg/ml)

Leave the bottom right well empty for orientation. Use a new yellow tip to scrape a bit of each white colony, then swirl it in a well. Gently shake the plate for 3-6 hours at 37°C. Parafilm/tape the lid closed and store at –80°C until use.

Transferring colonies to a membrane (Hedgehogging)

Cut a piece of membrane to fit the microtitre plate. Cut a separate membrane for each probe you intend to use. Cut off the bottom right corner and label the top left with pencil. Lie the membranes on a large plate containing L-agar with ampicillin at 100ug/ml. Prepare a bath of 100 % ethanol for sterilising the hedgehog (use a microtitre plate lid). Dip the hedgehog into ethanol, flame, then allow to cool. Dip cool hedgehog into thawed colonies in microtitre plate for a few seconds. Transfer to membrane, keeping the orientation the same –pressing gently to make sure all the spikes contact. When all done, put the lid on or cover large plate with alfoil and grow overnight at 37°C. If you don't have a hedgehog, you can do this step manually with yellow tips.

The next day you should see small yellowish colonies where the hedgehog contacted the membrane.

Lyse the colonies onto the membrane

Use forceps to place the membranes onto a piece of filter paper that has been pre-wetted with 2 x SSC/5% SDS for 2 minutes. Remove the membranes and microwave them on high for about 40 seconds or until dry (not burnt). This is best done on a clean paper towel. Wrap in gladwrap and store at room temp until use.

5. Radioactive Probing

Wash the membrane in 2 x SSC. Float for 2 minutes then sink and soak for a further 5 minutes.

Prehybridisation

For each probe make:

75ml 0.5M Na₂HPO₄

75mls MilliQ water

300µl EDTA

10.5g SDS (add last and wear a mask).

Warm it in the microwave to dissolve the SDS (about 1.5 min on medium).

Pour into your plastic Tupperware probing container. Put membranes in and pre-hyb for 1.5 hours at 65°C with v. low rocking. Also put in a small piece of blank membrane as a control. You can put multiple membranes on top of one another in a box –just make sure they get covered with the hyb mix.

Meanwhile,

Make your radioactive probes

I made these with the 'giant probes' and another time the 'lig-concatemers'. In the Armour et al paper, they found that large probes were more effective at revealing microsatellites than the standard 30mer oligos that people often use.

Dilute the giant probes or lig-concatemers probes to 5ng/µl.

You need a labelling kit (eg. Megaprime kit from Amersham).

Put 5µl of each giant probe into labelled 1.5 ml tubes plus 5µl of primer from the megaprime kit.

Denature by floating in boiling water for 5 minutes. Spin down.

Add 10 µl labelling buffer

Add 2 μ l klenow enzyme

Add 25 μ l milliQ water (total volume is now 50 μ l). Mix by flicking and spin down.

Behind screen add 3 μ l isotope to each tube and mix by pipetting (add more isotope if it's older).

Put the sample in a pre-warmed (37°C) lead pottle and incubate at 37°C for 1 hour.

Meanwhile,

Make columns (to use to separate unincorporated isotope).

You need 2 x 10ml tubes and, 2 x 1ml syringes without plungers per probe. Also, siliconised beads for each syringe.

Get a bead to sit at the bottom of the syringe, and the syringes to sit in the 10ml tubes. Use a disposable pipetter to fill the syringe with sephadex –avoiding air bubbles. When full, spin at ~2250 rpm for 4 minutes. Throw away the flow through and refill syringe with sephadex. Repeat this until only sephadex granules fill the syringe to 1ml. The columns are now ready.

Retrieve probe and add 50 μ l TE and 5 μ l 0.2M EDTA to each. Flick and spin down,

Put a lidless 1.5ml tube in the bottom of the 10ml tube. Load the probe into the top of the first column and spin @ 2250rpm for 4 minutes. Throw out syringe.

Pipette the flow through into the next column and repeat spin.

Pipette the flow through into a new, labelled 1.5 ml tube with a pierced lid. Float on boiling water for 5 minutes (in a fume-cupboard with a screen), then put on ice for 5 minutes.

Add the probes to the hyb boxes and leave overnight at 65°C with v. gentle rocking. A Geiger-counter set at 100x should indicate that the boxes are HOT. Make sure the boxes are well sealed.

Rinsing membranes

Make a 2x SSC/0.1% SDS wash solution. Warm it to 65°C. When ready, pour the probes into schott bottles and store behind a screen. Put the oven to 50°C. Pour wash solution into container and membranes and swish gently around for ca. 30 secs. Tip down the sink with plenty of water. Repeat. Pour remainder of wash solution into containers and put back in oven @ 50°C for 15 minutes.

Tip wash down the sink. Check the blank membrane with the Geiger counter on low setting (0.1x) –it should be blank. Pat membranes dry.

Prepare membranes for film

On a cutting board behind a screen wrap each membrane in saran wrap separately and avoiding airbubbles. Leave about 1cm saran wrap around edges. Lie the membranes colony side up in a x-ray cassette with a screen. Map the positions of membranes. When all ready, take cassette and box of film to dark room.

Under red light cut bottom right corner of film for orientation. Lay the film over the membranes. Close and put in -80°C freezer overnight.

Next day, put the cassette in 37°C room until it thaws out.

Develop the film

Under red light fix clips to one edge of the film. Dip it into developer and shake gently for 3 minutes.

Wash with water

Dip into fixer for 2 minutes

Wash with running water for ~10 minutes and hang to dry. Lights on. You should see black dots where positive colonies are.

** On average, one third of my colonies came up positive for microsatellite inserts.**

Growing and sequencing positives

Use a grid to work out which colonies are positive. Thaw out the microtitre plate and swirl a yellow tip in each positive. Drop the tip into a 10ml tube containing 3mls of L-broth with 0.1mg/ml ampicillin. Put alfoil over the top (allow air to circulate). Grow with 500rpm shaking at 37°C overnight. Also run a tube containing a blank yellow tip.

Miniprep and sequence positive clones.

Appendix.

Useful solutions.

20 x SSC

175.3g Sodium chloride

88.2g Sodium citrate

Make to 800ml and pH with 10M NaOH.

Fill to 1 litre.

Church and Gilbert solution

75ml 1M Disodium hydrogen orthophosphate

300µl 0.5M EDTA

10.2ml 10% SDS

64.5ml milliQ water

SOC buffer

2g bactotryptone (= bactopectone)

0.5g bacto yeast

1.0ml 1M NaCl

0.25ml 1M KCl

1ml 2M Mg²⁺ stock solution (filter sterilised)

1ml 2M glucose (filter sterilised)

Mix the first 4 ingredients and fill to 98mls with milliQ water. Autoclave and cool.

Add Mg⁺ and glucose.

L-Broth (L-agar is the same but add 15g agar –and mix on heat to dissolve)

10g peptone

5g yeast extract

10g NaCl

make up to 1L with dH₂O

Divide into 300ml aliquots and put each into a 1L bottle.

Autoclave.

0.5M EDTA pH 8.0

18.61g EDTA
80ml milliQ water
pH to 7.0 with NaOH pellets
then pH to 8.0 with 10M NaOH solution
Make up to 100mls and check pH

A list of chemicals/enzymes required

<i>Taq</i> , MgCl ₂ PCR buffer and dNTPs	<input type="checkbox"/>
Enzyme (NdeII=MboI=SAU3A)	<input type="checkbox"/>
Agarose etc	<input type="checkbox"/>
Linker DNA (order these)	<input type="checkbox"/>
T4 DNA ligase (plus buffer)	<input type="checkbox"/>
Oligos of the chosen repeat motifs (order these)	<input type="checkbox"/>
T4 PNK (= phosphonucleokinase)	<input type="checkbox"/>
Gel DNA recovery kit (eg. Highpure -Roche)	<input type="checkbox"/>
2M NaOH	<input type="checkbox"/>
20mM EDTA	<input type="checkbox"/>
1M Tris HCL pH 4.8, also pH 7.5	<input type="checkbox"/>
Church and Gilbert solution	<input type="checkbox"/>
20 x SSC buffer	<input type="checkbox"/>
10 % SDS	<input type="checkbox"/>
1M KOH	<input type="checkbox"/>
Glycogen	<input type="checkbox"/>
NaOAc pH. 5.2	<input type="checkbox"/>
100 % ethanol, 70% ethanol	<input type="checkbox"/>
pUC18 plasmid cut with Bam H1 and cap'ed	<input type="checkbox"/>
Max efficiency DH5 α cells (eg. Gibco).	<input type="checkbox"/>
SOC buffer	<input type="checkbox"/>
L-broth/L-agar	<input type="checkbox"/>
Ampicillin	<input type="checkbox"/>
Glycerol	<input type="checkbox"/>
0.5 M Na ₂ HPO ₄	<input type="checkbox"/>
Isotope, film, labelling kit, sephadex to make columns (for radioactive work - if applicable)	<input type="checkbox"/>