Microsatellite Libraries by Enrichment.

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Version 1.0, September 2001.

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This protocol was developed by Faye Barker and Paul Bloor as part of their respective PhD projects. This document was assembled by Phill Watts who has used it to guide a succession of undergraduate students with spectacular success. It derives from a mix of protocols described in the references on the next page. We use Qiagen kits and Promega vector cloning kits - we *strongly* recommend that you read these manuals before using the kits. Alternative kits may work just as well.

We hope it works as well for you - If you publish work using this protocol, please cite it.

Any comments or suggestions should be sent to Steve Kemp [kempsj@liv.ac.uk]

References:

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Materials required:

Glucose,
isopropanol, LA, LB,
$MgCl_2.6H_20$, $MgSO_4.7H_2O$,
SOC-medium, 20 X SSC, 6 X SSC, 2 X SSC, 1 X SSC, Tris-HCl (10 mM, pH 7.5),
1 X Washing/Binding (W/B) buffer (1 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA),
2 X W/B buffer (1 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA),

ABgene:	Reddy-Mix
AnalaR:	100 % glycerol
BDH:	PCR-grade water
Boehringer-Mannheim:	*Sau*3AI
Dynal:	M2-80 Dynabeads: Streptavadin-coated magnetic beads
FMC BioProducts:	NuSieve GTG agarose
Melford Laboratories:	X-gal, IPTG
Millipore:	Microcon YM-100 / YM-50 spin column
MWG-Biotech:	Oligonucleotide A, PO_4-Oligonucleotide B, 3'-biotinylated oligonucleotide, 50 X UV-safe TAE buffer
New England Biolabs:	*Alu*I, *Hae*III
Promega:	*Rsa*I, 100 bp PCR ladder, 2 X T4 DNA Rapid ligation buffer, T4 DNA ligase, pGEM®-T vector, JM109 High Efficiency Competent Cells
Qiagen:	QIAquick gel extraction kit, MinElute PCR purification kit
Sigma:	ethidium bromide, ampicillin, N,N'-dimethyl-formamide

Suggested Timetable:

		To do:	Approx. time:	To make:
Day 1	**am**	Dilute oligonucleotides, Quantify genomic DNA.	30 min 45 min	2 % agarose gel, 20 X SSC, 6 X SSC,
	pm	**2.** Digest genomic DNA, Check digestion on 2 % agarose gel.	3 hr 45 min	2 X SSC, 1 X SSC, W/B Buffer.
Day 2	**am**	**1.** Anneal oligonucleotides,	1.5 hr	
	pm	**2.** Ligate adaptors to digested DNA.	30 min (o/night)	1.8 % NuSeive gel
Day 3	**am**	**3.** Size-select 400-1,000 bp fragments on 1.8 % Nuseive gel, Purify fragments from NuSeive gel,	1.5 hr 45 min	2 % agarose gel, Ampicillin.
	pm	PCR-test ligation success and size, Check PCR on 2 % agarose gel.	30 min (3 hr) 45 min	Agar plates, SOC medium.
Day4	**am**	**4.** Enrich and capture DNA.	2 hr	2 % agarose gel
	pm			
Day 5	**am**	**4*xxi*.** PCR enriched DNA fragments.	30 min (3 hr)	IPTG.
	pm	**5.** Clean-up PCR, Test PCR success on 2 % agarose gel, **6*a*.** Ligation into pGEM®-T vector.	45 min 45 min 30 min (o/night)	X-gal.
Day 6	**am**	**7b.** Transformation into *E.coli*, Incubate competent cells.	1 hr 3 hr	IPTG/X-gal plates.
	pm	Plate out colonies and incubate overnight.	45 min	96-well 2 % agarose gel.
Day 7	**am**	**8*a*.** Pick positive clones, (**9.** PCR-screen positive clones).	upto 8 hr	30 % glycerol/LB, (Stabs).
	pm	(plate-out microsatellite-containing clones)	1 hr	
Day 8	**am**	Construct back-up/storage library, (Prepare stabs).	upto 5 hr 1 hr	
	pm			

Notes:

- **Steps 4*xxi*-6*a* *must* be completed during the same day.**

1. Adaptor preparation:

(***i***) Mix the following components in a 0.5 ml microcentrifuge tube:

10 µl Oligonucleotide A (200 pmol/µl)
10 µl Oligonucleotide B (200 pmol/µl)

(***ii***) Mix oligos by gentle pipetting or vortexing, then spin briefly.
(***iii***) Denature oligo mixture by heating at 80ºC for 2-5 min on a thermocycler.
(***iv***) Allow oligos to anneal by leaving to cool at room temperature for 1 hour.
(***v***) Add 60 µl of PCR-grade water (BDH). [*i.e.* make up to a *total* of 80 µl]
(***vi***) Store adapter mixture at -20ºC.

Sticky end adaptor (*Sau*3A1-specific).

		5’	3’	
Oligo A		GGC CAG AGA CCC CAA GCT TCG		[21-mer]
Oligo B	PO_4 -	GAT CCG AAG CTT GGG GTC TCT GGC C		[25-mer]
Adaptor		GGC CAG AGA CCC CAA GCT TCG CCG GTC TCT GGG GTT CGA AGC CTA G - PO_4		[*Sau*3A1 restriction site]

Blunt end adaptor (*e.g.* *Hae*III / *Alu*I / *Rsa*I).

		5’	3’	
Oligo A		CTC TTG CTT ACG CGT GGA CTA		[21-mer]
Oligo B	PO_4 -	TAG TCC ACG CGT AAG CAA GAG CAC A		[25-mer]
Adaptor		CTC TTG CTT ACG CGT GGA CTA A CAC GAG AAC GAA TGC GCA CCT GAT - PO_4		

Notes:

- Dilute oligonucleotides with PCR-grade water, leave to dissolve at 4ºC for 2 hours to overnight, then make aliquots. Store oligonucleotide aliquots and stocks at -20ºC.
- The adaptor consists of two oligonucleotides with complementary sequences that are heated and allowed to anneal together.
- Oligo B has a phosphate-group (PO_4) at the 5’ end and an extra four bases at its 3’ end.

2. Digestion of genomic DNA and adapter ligation:

The importance of using high-quality genomic DNA for the construction of a genomic library *cannot* be emphasised enough - problems with sequencing likely happen if poor-quality DNA is initially used. It is also recommended that a test digest with several different enzymes is performed on *ca*. 1 µg of DNA to determine the restriction enzyme that provides the best size-distribution of DNA fragments.

All frozen components should be thawed on ice. Always add the restriction enzyme or T4 DNA ligase ***last***, removing it from -20ºC when needed and return the enzyme to -20ºC ***immediately***.

(***i***) Mix the following components in a 0.5 ml microcentrifuge tube:

. µl	genomic DNA	[9 µg]
9 µl	10 X enzyme buffer	[final concentration 1 X]
. µl	restriction enzyme	[40 U]
. µl	PCR-grade water (BDH)	
90 µl	**total volume**	

(***ii***) Incubate at 37ºC for 1.5 - 2 hr.
(***iii***) Inactivate the enzyme by heating it to 65ºC for 5-10 min.
(***iv***) To the digest add:

100 µl	2 X T4 DNA ligase buffer	[final concentration 1 X]
. µl	PCR-grade water (BDH)	
2.0 µl	adaptor	[25 pmol/µl]
2.0 µl	T4 DNA ligase (Promega)	[40 U]
200 µl	**total volume**	

(***v***) Mix gently by pipetting and incubate at either (***a***) 4ºC overnight or (***b***) room temperature for 1 hr.
(***vi***) Inactivate the enzyme by heating to 65ºC for 5-10 min.

(***vii***) If several samples are being digested (*e.g*. with more than one restriction enzyme) then the digested DNA can be pooled and concentrated using a Microcon YM-50 spin column (Millipore) if the total volume is too great.

(***viii***) Test the success of the digestion on a 2 % agarose gel made with ethidium bromide (Sigma) at a final concentration of 0.5 µg/ml.

Notes:

- Suitable restriction enzymes include:
  1. *Sau*3A1 for the sticky-end adaptor.
  2. *Hae*III, *Alu*I and *Rsa*I for blunt-end adaptor.
- All restriction enzymes used in this protocol recognise a 4 bp restriction site - on average they are expected to cleave DNA every 256 bp.
- Using several blunt-end restriction enzymes and pooling the digestion product may increase the number of different fragments that contain microsatellites.

3. *Size-selection and PCR of adaptor-ligated DNA:*

(***i***) Adaptor-ligated DNA is run (*ca*. 1 hour at 60 v) alongside a 100 bp PCR ladder (Promega) in a 1.8 % NuSieve GTG (FMC BioProducts) agarose gel prepared with UV-safe TAE buffer (MWG Biotech) and ethidium bromide (Sigma) at a final concentration of 0.5 µg/ml.
(***ii***) Visualise gel using a uv-transilluminator (**Figure 1**) and quickly (< 20 s) excise the 400-1,000 bp fragments (**Figure 2**) using the blunt end of a sterile scalpel blade (or a microscope cover-slip). Put the gel slices into a ***pre-weighed*** 1.5. ml centrifuge tube.

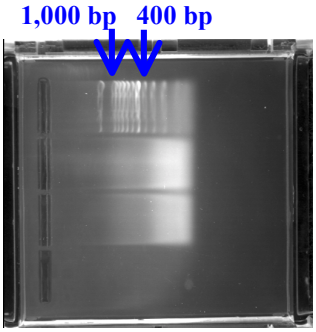

Figure 1. Digested genomic DNA alongside DNA ladder

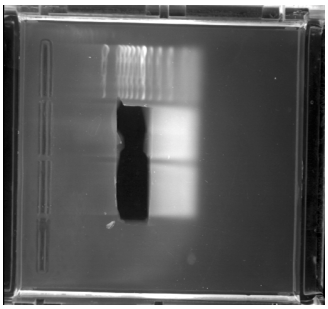

Figure 2. Removal of 1,000-400 bp fragments.

(***iii***) Purify the DNA fragments using a QIAquick gel extraction kit (Qiagen):
(***iv***) Add 3 volumes of buffer QG to 1 volume of gel (100 mg ~ 100 µl).
[maximum of 400 mg per QIAquick column]
(***v***) Incubate at 50ºC for 10 min (or until the gel slice has completely dissolved). *Briefly* (1-2 sec) vortex the tube every 2-3 min to help dissolve the sample.
(***vi***) After the gel has dissolved check that the mixture is yellow.
[If the colour is orange/violet then add 10 µl of 3 M sodium acetate pH 5.0 and mix - the mixture will turn yellow]
(***vii***) Place QIAquick spin column in a 2 ml collection tube, apply sample to QIAquick column and centrifuge at room temperature for 1 min.
(***viii***) Discard flow-through and replace QIAquick column in the same 2 ml collection tube.
(***ix***) ***[optional]*** Add 0.5 ml buffer QG to QIAquick column and centrifuge for 1 min.
(***x***) (Discard flow through) Add 0.75 ml buffer PE to QIAquick column, let stand for 2-5 min and centrifuge for 1 min.
(***xi***) Discard flow-through and centrifuge the QIAquick for an additional 1 min at 13,000 rpm.

Notes:

- A 1.8 % agarose gel contains 0.54 g agarose in 30 ml buffer.
- Use a large-well comb so that enough digested DNA can be loaded into a single well.
- To prevent damage to the (*expensive*) uv-transilluminator keep the gel in the (*cheap*) casting tray when excising the fragments.
- 400-1,000 bp fragments are excised since fragments over *ca*. 1,000 bp long are unlikely to be completely sequenced, and sequences smaller than 400 bp are rarely long enough to provide good primers.

3. Size-selection and PCR of adaptor-ligated DNA (cont.):

(***xiv***) Place the QIAquick column into a clean 1.5 ml centrifuge tube.
(***xv***) Elute the DNA fragments in 30 µl of 10 mM Tris-HCl (pH 8.5): apply elution buffer to centre of QIAquick column, let stand for 1 min and centrifuge at 13,000 rpm for 1 min.
[Buffer EB in Qiagen kits]
(***xvi***) Check the yield of recovered DNA on a 2 % agarose gel.
[approximately 4 µg of DNA is expected]
(***xvii***) Pool all recovered DNA samples and reduce volume to *ca*. 10 µl using a Microcon YM-100 (or YM-50) spin column (Millipore).
(***xviii***) Perform a PCR to test the success of the ligation of adaptors to the digested DNA; combine the following:

1.25 µl	Oligonucleotide A (200 pmol/µl)
10.25 µl	PCR-grade water (BDH)
1 µl	size-selected DNA
12.5 µl	Reddy-Mix (ABgene)
25 µl	**total volume**

(***xix***) Cycle the PCR mix on the following program:

Step 1	95ºC	5 min
Step 2	95ºC	50 s
Step 3	56ºC	1 min
Step 4	72ºC	2 min
Step 5	go to step 2 thirty times	
Step 6	72ºC	10 min
Step 7	6ºC	hold

[approximate time 2 hours]

(***xx***) Check PCR by running 5 µl on a 2 % agarose gel alongside a 100 bp DNA ladder (Promega). Successful ligation of adaptors to digested DNA is indicated by a smear between 400-1,000 bp.

Notes:

- A 2 % agarose gel contains 0.6 g agarose in 30 ml buffer.
- Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA.
- Elution efficiency is dependent on pH: maximum efficiency is achieved between pH 7.0 and 8.5.
- New Qiagen columns are available that elute in a 10 µl volume.

4. Capture of microsatellite-containing DNA fragments:

(***i***) In a 1.5 ml microcentrifuge tube, wash 100 µl of streptavadin-coated magnetic beads (10 mg/ml) (M-280 Dynabeads, Dynal) in 100 µl of 1 X Washing/Binding (W/B) buffer (1 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA).
(***ii***) Repeat (***i***) above.
(***iii***) Re-suspend the beads in 200 µl of 2 X W/B buffer.
(***iv***) Add 2 µl (200 pmol) of 3'-biotinylated oligonucleotide (at 100 mM).
(***v***) Make the total volume to 400 µl with PCR-grade water (BDH).
(***vi***) Incubate the sample at room temperature for 30 min, with gentle agitation every 10 min.
(***vii***) Wash once in 400 µl of 1 X W/B and twice in 400 µl of 6 X SSC.
(***viii***) Re-suspend the beads in 50 µl 6 X SSC and incubate at probe-specific hybridisation temperature (**Table 1**).
(***ix***) In a *separate* tube add:

10 µl	digested/adaptor-ligated/size-selected DNA	
. µl	Oligonucleotide A (20 pmol)	
15 µl	20 X SSC	[final concentration 6 X]
. µl	PCR-grade water (BDH)	
50 µl	**total volume**	

(***x***) Mix by gentle pipetting and briefly spin.
(***xi***) Set up the following program on a thermal cycler:

Step 1	95ºC	5 min	
Step 2	60ºC	30 min	[probe-specific hybridisation temperature]
Step 3	70ºC	2 hr	

(***xii***) Incubate the DNA mixture (***step ix***) at 95ºC for 10 min.
(***xiii***) As the temperature ramps down to 60ºC add the contents of the re-suspended bead mixture (***steps i-viii***) to the DNA sample, mix by *gentle* pipetting and incubate at 60ºC for 30 min with gentle agitation every 5 min.
(***xiv***) Remove beads using a magnet and quickly remove the supernatent. Re-suspend the beads in 100 µl of 2 X SSC and transfer to a 1.5 ml microcentrifuge tube.

[It is *essential* that the beads *do not* dry out between washes]

(***xv***) Wash the bead mixture *four* times in 1 ml of 2 X SSC, incubating the sample for 5 min at room temperature before removing the 2 X SSC.
(***xvi***) Wash the bead mixture *four* times in 1 ml of 1 X SSC, incubating the sample for 5 min at room temperature before removing the 1 X SSC.

Notes:

- To wash the Dynabeads® (*i.e.* remove supernatent) place the tube next to a magnetic stand (or magnetic stirrer) for 2-3 min to allow the beads to migrate to the side of the tube. Using a pipet, remove the supernatent without moving the tube or touching the beads. Next remove the tube from the magnet and add buffer as required; the beads may need resuspension by ***gentle*** pipetting. Repeat the steps as required.
- Removing beads is achieved by placing the tube next to a magnet for 2-3 min so that the beads are separated from the supernatent.

4. Capture of microsatellite-containing DNA fragments (cont.):

(***xvii***) Re-suspend the bead mixture in 100 µl of 1 X SSC and aliquot into four 25 µl samples. Add 250 µl 1 X SSC to each aliquot and incubate at the probe-specific temperature for 10 min.
(***xviii***) Quickly remove the supernatent and rinse for 30 s at room temperature in 400 µl 1 X TE.
(***xix***) Rinse for 30 s at room temperature in 400 µl 50 mM NaCl.
(***xx***) Re-suspend each aliquot in 50 µl PCR-grade water (BDH).

[final bead concentration is 5 µg/µl]

(***xxi***) Set up the following PCR to increase the quantity of enriched DNA:

25 µl	2 X Reddy-Mix (ABgene)
3 µl	Oligonucleotide A (10 pmol/µl)
8 µl	bead suspension (40 µg)
14 µl	PCR-grade water (BDH)
50 µl	**total volume**

(***xxii***) Run the PCR on the following program:

Step 1	95ºC	3 min	
Step 2	95ºC	30 s	
Step 3	60ºC	30 s	[probe-specific annealing temperature]
Step 4	72ºC	45 s	
Step 5	go to step 2 five times		
Step 6	92ºC	30 s	
Step 7	60ºC	30 s	[probe-specific annealing temperature]
Step 8	72ºC	55 s	
Step 9	go to step 6 thirty times		
Step 10	72ºC	30 min	
Step 11	4ºC	hold	[approximate time 3 hours]

(***xxiii***) Run 2-5 µl of PCR product alongside a PCR marker (Promega) on a 2 % agarose gel at 100 V for 15-20 min. A smear between 0.4 - 1 Kbp indicates a successful capture and PCR.

Table 1. Hybridisation, washing and PCR temperatures for some oligonucleotides.

Probe	T_m / ºC	Hyb / ºC	PCR T_a/ ºC	Probe	T_m / ºC	Hyb / ºC	PCR T_a/ ºC
$(GT)_{15}$	82	75	65	$(AAT)_8$	56	50	40
$(GT)_{12}$	77	70	60	$(CAG)_8$	84	78	68
$(AAAG)_6$	77	67	57	$(GAA)_8$	80	70	60
$(GTG)_8$	94	84	64	$(AAC)_8$	70	64	54

Notes:

- Annealing temperature of probes may be calculated using the oligo calculator at: http://www.basic.nwu.edu/biotools/oligocalc.html.
- For PCR use an annealing temperature 5-10ºC below the annealing temperature calculated above.
- Proceed to the PCR clean-up and ligation steps immediately (or the next day at the latest!).

5. PCR clean up:

(***i***) Add 5 volumes of buffer PB to 1 volume of the PCR reaction and mix.
[It is not necessary to remove mineral oil]
(***ii***) Place a MinElute column in a 2 ml collection tube.
(***iii***) Apply the sample to the MinElute column and centrifuge for 1 min at 13,000 rpm.
(***iv***) Discard flow-through. Place the MinElute column back in the same 2 ml collection tube.
(***v***) Add 750 µl buffer PE to the MinElute column and centrifuge for 1 min at 13,000 rpm.
(***vi***) Discard flow-through and place the MinElute column back in the same collection tube. Centrifuge the column for 1 min at 13,000 rpm.
[Residual ethanol from buffer PE will not be removed unless the flow-through is discarded before this step]
(***vii***) Place the MinElute column in a clean 1.5 ml centrifuge tube.
(***viii***) To elute the DNA, add 10 µl buffer EB (or 10 mM Tris-HCl, pH 8.5) to the centre of the membrane, let the column stand for 1 min and centrifuge for 1 min at 13,000 rpm.

Notes:

6a. Ligation of enriched DNA into pGEM®-T vector.

(***i***) Briefly centrifuge the pGEM®-T vector to collect the contents at the bottom of the tube.
(***ii***) Vortex the 2 X Rapid ligation buffer vigorously before each use.
(***iii***) Using 0.5 ml centrifuge tubes with low DNA-binding capacity set up the following ligation reaction:

5 µl	2 X Rapid Ligation Buffer (Promega)
1 µl	pGEM®-T vector (50 ng) (Promega)
x µl	PCR product (***see section 6b below***)
1 µl	T4 DNA ligase (3 Weiss units/µl)
x µl	PCR-grade water (BDH)
10 µl	**total volume**

(***iv***) Mix the reactions by pipetting.
(***vi***) Incubate the reaction for either
(***a***) 1 hr at room temperature, or
(***b***) overnight at 4ºC. [this provide the maximum number of transformants]

6b. Optimising insert:vector molar ratios.

(***i***) pGEM®-T vector was optimised using a 1:1 molar ratio of insert DNA to vector - ratios of 1:8 to 8:1 can be used.
(***ii***) Ratios of 3:1 to 1:3 are typically used initially.
(***iii***) pGEM®-T vector is approximately 3 Kb long.
(***iv***) To calculate the appropriate amount of PCR product (insert) to use in the ligation reaction use the following equation:

$$\frac{\text{ng vector} \times \text{Kb size of insert}}{\text{Kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

Notes:

- Use the Promega T4 DNA ligase supplied with the pGEM®-T vector kit.
- 2X rapid ligation buffer contains ATP which degrades during temperature fluctuations; avoid multiple freeze-thaw cycles by making single-use aliquots.
- ***IMPORTANT:*** Vortex the 2X rapid ligation buffer before each use.
- Longer incubation times will increase the number of transformants.
- For overnight ligations, float the tubes in polystyrene on a bath of room-temperature water and place the bath in 4ºC refrigerator.

7a. Preparation of agar plates.

(***i***) LB/ampicillin plates: Melt 400 ml LA in microwave (at *defrost* for 15-20 min). Allow LA to cool to 50ºC before adding ampicillin to final concentration of 100 µg/ml. Pour medium (*ca*. 30-35 ml) into petri-dishes and allow to harden. Store plates at 4ºC (for up to 1 month).
(***ii***) LB/ampicillin/IPTG/X-gal plates: Spread 100 µl of 0.1 M IPTG and 20 µl of 50 mg/ml X-gal over the surface of an LB/ampicillin agar plate; allow to absorb for at least 30 min at 37ºC prior to use.
(***ii***) LB/ampicillin/IPTG/X-gal plates: [***Optional***] Make LB/ampicillin plates as above but add 0.5 mM IPTG and 80 µg/ml X-gal before pouring plates.

7b. Transformation of pGEM®-T vecor into E. coli.

(***i***) Prepare LB/ampillicin/IPTG/X-gal plates as described above. Equilibrate plates to room temperature prior to plating.
(***ii***) Centrifuge tubes containing ligation reaction to collect contents at bottom of tube.
(***iii***) Add 2 µl of each ligation reaction to a 1.5 ml centrifuge tube on ice.
(***iv***) Remove tube(s) of frozen JM109 High Efficiency Competent Cells (from -70ºC storage) and place in an ice bath until *just* thawed (*ca*. 5 min). Mix the cells by ***gently*** flicking the tube.
(***v***) ***Carefully*** transfer 50 µl of cells into each tube prepared in step 2.
(***vi***) ***Gently*** flick the tubes to mix and place them on ice for 20 min.
(***vii***) Heat-shock the cells for 45-50 sec in a water bath at ***exactly*** 42ºC - ***DO NOT SHAKE***.
(***viii***) Immediately return the tubes to ice for 2 min.
(***ix***) Add 950 µl room temperature SOC medium to the tubes containing cells transformed with ligation reactions. [LB broth may be substituted, but colony number may be lower]
(***x***) Incubate for 1.5 hr at 37ºC with shaking at approximately 150 rpm.
(***xi***) Plate 100 µl of each transformation culture onto LB/ampicillin/IPTG/X-gal plates.
(***xii***) Incubate the plates overnight (16-24 hr) at 37ºC.
(***xiii***) ***[optional]*** incubation of plates at 4ºC (after 37ºC incubation) helps develop the blue/white colour of colonies.

Notes:

- Dissolve ampicillin in sterile distilled water to a final concentration of 50 mg/ml; store as aliquots at -20ºC. Adding ampicillin to LA before it cools below 50ºC destroys the ampicillin.
- Colonies generally grow better on plates that have been prepared a few days beforehand.
- Be *extremely* careful with competent cells - any vigorous treatment will destroy them.
- The use of 17x100 mm polypropylene tubes (Falcon # 2059) usually increases transformation efficiency - tubes from some manufacturers bind DNA and decrease colony number.

8a. Working library construction:

(***i***) Add ampicillin to LB to a final concentration of 100 µg/ml.
(***ii***) Pipette 100 µl of LB/ampicillin into each well of as many 96-well plates as required.
(***iii***) Using a sterile toothpick or a 200 µl pipet tip (one for each colony), pick a colony containing an insert and swirl tip into a well; repeat until all colonies have been picked making sure that only one colony is added to each well.
(***iv***) Incubate 96-well plates at 37ºC for 3-4 hours.
(***v***) Store plates at 4ºC for up to 3 weeks.

8b. Long-term storage library construction:

(***i***) Prepare glycerol stock medium by adding 30 ml 100 % glycerol (AnalaR) to 70 ml LB.
[final glycerol concentration of 30 %]
(***ii***) Autoclave 30 % glycerol stock.
(***iii***) Add ampicillin to LB to a final concentration of 100 µg/ml.
(***iv***) Pipette 100 µl of LB/ampicillin into each well of as many 96-well plates as required.
(***iv***) Using sterile 1-10 µl pipet tips transfer 10 µl of the working library into corresponding wells of the back-up plates.
(***v***) Incubate 96-well plates at 37ºC for 3-4 hours.
(***vi***) Add 100 ml of 30 % glycerol stock to each well.
(***vii***) Store at -80ºC. [Bacterial cultures may be stored indefinitely in glycerol cultures]

8c. Stab construction:

(***i***) Melt LA in microwave (15-20 min on defrost).
(***ii***) Add ampicillin to LB to a final concentration of 100 µg/ml when LB is at 50ºC or lower.
(***iii***) Use 2 ml plastic vials with screw caps fitted with rubber gaskets.
(***iv***) Add 1.5 ml molten LA/ampicillin and let cool with vial caps loose.
(***v***) Tighten vial caps when the agar has set.
(***vi***) To store bacteria, pick a single, well isolated colony with a sterile loop.
(***vii***) Stab the loop through the agar to the bottom of the vial.
(***viii***) Replace and tighten the cap. Label the vial and cap.
(***ix***) Store the vial in the dark at room temperature.

Notes:

- White colonies generally contain inserts, but see notes in the Promega pGEM®-T vector cloning kit manual.

9a. PCR screening for microsatellite-containing clones.

(***i***) Prepare the following master mix:

5 µl	Oligo A (10 pmol/µl)
2.5 µl	$(GT)_{15}$ oligonucleotide (10 pmol/µl)
2.5 µl	PCR-grade water (BDH)
10 µl	2 X Reddy Mix (ABgene)
20 µl	**total volume**

(***ii***) Pipette 20 µl of the PCR master mix into each well of 96-well PCR plate.
(***iii***) At the same time as **stage 8*a*** place the toothpick/pipet tip into a well corresponding to the library plate and swirl to release some cells.
(***iii***) Run the PCR on the following PCR program (SCRGT):

Step 1	95ºC	3 min	
Step 2	95ºC	30 s	
Step 3	60ºC	30 s	[probe-specific annealing temperature]
Step 4	72ºC	45 s	
Step 5	go to step 2 five times		
Step 6	92ºC	30 s	
Step 7	60ºC	30 s	[probe-specific annealing temperature]
Step 8	72ºC	55 s	
Step 9	go to step 6 thirty times		
Step 10	72ºC	30 min	
Step 11	4ºC	hold	[approximate time 3 hours]

(***iv***) Load 5-10 µl of PCR product alongside a PCR marker (Promega) on a 2 % agarose gel containing ethidium bromide (Sigma) at a final concentration of 0.5 µg/ml. Run the gel at approximately 100 V for about 15-20 min.
(***v***) A double banded PCR product indicates that the vector contains a microsatellite-containing insert (**Figure 3**).

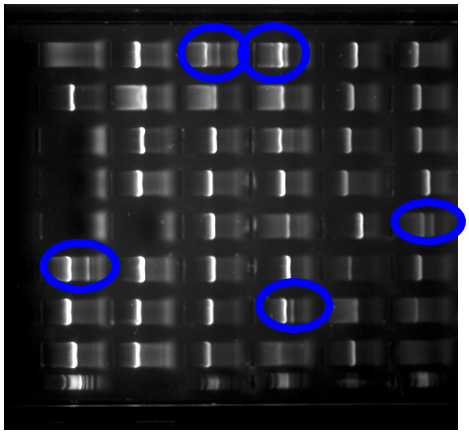

Figure 3. PCR screening of clones. Blue circles indicate a microsatellite.

Notes:

APPENDIX: Commonly used buffers and solutions.

Ampicillin: Dissolve 50 mg in 1 ml H_2O and filter sterilize. Store in 1 ml aliquots at -20ºC.

0.5 M EDTA: Add 186.1 g disodium ethylenediaminetetra-acetate.2 H_2O (EDTA) to 800 ml H_2O. Stir vigorously on a magnetic stirrer. Adjust pH to 8.0 with NaOH (*ca*. 20 g NaOH pellets). Sterilise by autoclaving.

Ethidium Bromide: Add 1 g ethidium bromide to 100 ml of H_2O and stir on magnetic stirrer for several hours. wrap the container in aluminium foil and stor at room temperature. The solution concentration is 10 mg/ml.

0.1 M IPTG: Dissolve 1.2 g IPTG in water to 50 ml final volume. Filter sterilise and store at -20ºC.

LA: Prepare LB as below. Just before autoclaving add 15 g (per litre) of bacto-agar.

LB medium: To 950 ml H_2O add 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl. shake the solution until solutes dissolve and adjust the volume to 1 litre. Sterilise by autoclaving.

2 M Mg^{2+}: Mix 20.33 g $MgCl_2.6H_20$ and 24.65 g $MgSO_4.7H_2O$ with distilled water to 100 ml; filter sterilise and store at 4ºC.

1 M NaCl: Dissolve 58.44 g of NaCl in 1 litre of H_2O.

50 mM NaCl: Dissolve 2.922 g of NaCl in 1 litre of H_2O or dilute 1 M NaCl.

SOC: : Dissolve 2.0 g Bacto®-tryptone, 0.5 g Bacto®-yeast extract, 1 ml 1 M NaCl, 0.25 ml 1 M KCl, 1 ml 2 M Mg^{2+} stock, 1 ml 2 M filter-sterilised glucose. Store at 4ºC.

20 X SSC: Dissolve 175.3 g NaCl and 88.2 g sodium citrate in 800 ml of H_2O. Adjust to pH 7.0 with a few drops of a 10 N solution of NaOH. Make volume to 1 litre with H_2O and autoclave.

50 X TAE: Dissolve 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) in 1 litre of water. *Note*: this solution is 50 X - dilute 49:1 for working concentration.

1 M Tris: Dissolve 121.1 g of Tris base in 800 ml of H_2O. Adjust pH to desired value (*e.g*. 8.5 or 7.4) with HCl at room temperature; adjust the final volume to 1 litre with H_2O. Aliquot and sterilise by autoclaving. Other Tris buffers can be prepared by adjusting an aliquot of 1 M Tris with an appropriate volume of water.

1 X W/B buffer: Dissolve 5.844 g NaCl, 37 mg EDTA in 50 ml 20 mM Tris-HCl pH 7.5. Make volume up to 100 ml with distilled water. The final concentration of the solution is: (1 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA).

2 X W/B buffer: Dissolve 11.688 g NaCl, 37 mg EDTA in 50 ml 20 mM Tris-HCl pH 7.5. Make volume up to 100 ml with distilled water. The final concentration of the solution is: (2 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA).

X-gal: Dissolve 100 mg 5-bromo-4-chloro-3-indolyl-β-D-galactosidase in 2 ml N,N'-dimethyl-formamide. Cover with aluminium foil and store at -20ºC. The final concentration of X-gal is 50 mg/ml.