

Protocol for MIAMI (for comparison of sample 1 and sample 2) V4

Revised May 18, 2007

Amplification of DNA

Day1

(1) Make following mixtures (1A, 2A, 1B, 2B) by assembling the following components and mixing gently.

	1A, 2A	
Genomic DNA (sample1, 2)	500 ng	
10 x T*	10 ul	
0.1% BSA	10 ul	
<i>Hpa</i> II	40 units	/ to 100 ul with DW

	1B, 2B	
Genomic DNA (sample1, 2)	500 ng	
10 x T*	10 ul	
0.1% BSA	10 ul	
<i>Msp</i> I	40 units	/ to 100 ul with DW

*10 x T:

330 mM Tris-acetate (pH7.9)

100 mM Mg-acetate

5 mM Dithiothreitol

660 mM K-acetate

(2) Take out 4.5 ul of each mixture and put into new tubes. Add 0.5 ul (0.1ug) of control plasmid (ex. pBluescript) to these tubes and mix gently. These are used for

controls of digestion.

(3) 37°C overnight for mixture made in (1) and (2).

Day2

(4) Run 3 ul of controls in a 2% agarose gel and check for complete digestion.

(5) Phenol-Chlorform extraction of 1A, 2A, 1B, 2B.

(6) Recover aqueous phase and add 2 ul of Ethachinmate (Nippongene: 318-01793, This is a carrier for ethanol precipitation.) and mix.

(7) Ethanol precipitation.

(8) 70% Ethanol wash.

(9) Solved in 5 ul of TE.

(10) Add 1 ul of the adaptor* (400 pm/ul) and mix gently.

*Making by annealing of AGCACTCTCCAGCCTCTCACCGAG and CGCTCGGTGA in 20 mM Tris-HCl (pH8.3), 100 mM KCl

(11) Make ligation mixture as follows and mix gently.

10 x E coli Ligaton B*	5 ul		
E coli DNA Ligase (60 u/ ul)	5 ul		
DW	10 ul	/	20 ul

*10 x E coli Ligaton B:

300 mM Tris-HCl (pH8.0)

40 mM MgCl₂

10 mM Dithiothreitol

260 uM NAD⁺

500 ug/ ml BSA

(12) Add 4 ul of ligation mixture and mix gently.

(13) 16°C overnight.

Day3

(14) Make PCR mixture as follows and mix gently.

2 x GCB I*	220 ul
2.5 mM dNTP	70.4 ul
DW	134.2 ul
Primer**	4.4 ul
GeneTaq***	2.2 ul / 431.2 ul

*2 x GCB I: A good buffer for amplification of GC rich sequences (Takara: 9154)

** AGCACTCTCCAGCCTCTCACCGAG

*** GeneTaq (NipponGene: 312-02874).

(15) Prepare four new tubes with 98 ul of PCR mixture and name them as P1A, P2A, P1B, and P2B.

(16) Add 2 ul of following DNA and mix gently.

P1A: 1A

P2A: 2A

P1B: 1B

P2B: 2B

(17) PCR cycling as follows (Use GeneAmp PCR System 9700)

Mode: Max mode

Cycle: 72 °C, 5 min – 94 °C, 3 min

(94 °C, 10 sec - 70 °C, 30 sec - 72 °C, 2 min 30 sec) x 5 cycles

72 °C, 7 min

(18) Room temperature for 10 min.

(19) Add 50 units of *Msp* I and mix gently.

(20) Take out 4.5 ul of each mixture and put into new tubes. Add 0.5 ul (0.1ug) of control plasmid (ex. pBluescript) to these tubes and mix gently. These are used for controls of digestion.

(21) 37°C 3 hr for P1A, P2A, P1B, and P2B and controls.

(22) Run 3 ul of controls in a 2% agarose gel and check for complete digestion.

(23) PCR cycling as follows (Use GeneAmp PCR System 9700)

Mode: Max mode

Cycle for P1A and P2A:

(94 °C, 10 sec - 70 °C, 30 sec - 72 °C, 2 min 30 sec) x 13* cycles

72 °C, 7 min

Cycle for P1B and P2B:

(94 °C, 10 sec - 70 °C, 30 sec - 72 °C, 2 min 30 sec) x 10* cycles

72 °C, 7 min

*The number of cycle should be optimized for just before saturation.

(24) Run 2 ul in 2% agarose gel and check for amplification.

(25) Purify PCR amplified samples with MinElute PCR Purification kit (QIAGEN: 28004) and eluted with 11 ul TE.

(26) Use 1 ul for estimation of concentration.

(27) Dilute to 100 ng/ ul with TE.

Labeling and hybridization

Day1

(1) Make mixture as follows and mix gently.

	L1A	L2A	L1B	L2B
2.5 x random prime sol*	10 ul	10 ul	10 ul	10 ul
P1A	8 ul			
P2A		8 ul		
P1B			8 ul	
P2B				8 ul / 18 ul

*A component of BioPrime DNA Labeling System (Invitrogen, Cat No.:18094-011).

(2) 95 °C, 5 min.

(3) On ice.

(4) Add followings and mix gently.

	L1A, L1B	L2A, L2B
Cy3-dCTP*	1.5 ul	
Cy5-dCTP**		1.5 ul
dNTP***	4.5 ul	4.5 ul
Klenew****	1 ul	1 ul / 25 ul

*Cy3-dCTP (PA53021, AmershamPharmacia)

**Cy5-dCTP (PA55021, AmershamPharmacia)

***dNTP (1/3 mM dCTP, 2 mM dAGTTP, 1 mM Tris)

100 mM dCTP	3 ul		
100 mM dATP	18 ul		
100 mM dGTP	18 ul		
100 mM dTTP	18 ul		
1M Tris (pH7.5)	0.9 ul		
DW	842.1 ul	/	900 ul

****A component of BioPrime DNA Labeling System (Invitrogen, Cat No.:18094-011).

(5) 37 °C, 1 hr (shield the light).

(6) Add Stop buffer* and mix.

*A component of BioPrime DNA Labeling System (Invitrogen, Cat No.:18094-011).

(7) Combine and rename following samples.

A: L1A and L2A

B: L1B and L2B

(8) Pipette solution to sample reservoir of Microcon YM-30 (Millipore: 42410).

(9) Spin 4 min at 8,000 rpm with microcentrifuge at room temperature.

(10) Discard flow-through. Dilute concentrated solution in sample reservoir with 250 ul of TE.

(11) Spin 12 min at 8,000 rpm with microcentrifuge at room temperature

(12) Repeat (10)(11) two times (final spinning is extended to 20 min).

(13) Place sample reservoir upside down in a new tube, spin 2min at 8,000 rpm with microcentrifuge at room temperature to recover the labeled sample.

(14) Combine followings and gently mix (following protocol is for Agilent ChIP on CHIP microarray).

Labeled sample (A or B)	ul
10 x Blocking Agent*	50 ul
2 x Hybridization Buffer*	250 ul / to 500 ul with DW

* A component of Agilent oligo aCGH Hybridization kit (Agilent: 5188-5220).

(15) 95 °C, 3 min.

(16) On ice.

(17) A and B are independently hybridized to different microarrays according to Agilent instruction.

(18) Hybridization at 65 °C for 20 hr with rotation at 10 rpm.

Day2

(19) Take out the microarrays from the chamber in Wash Buffer 1 (Agilent: 5188-5221, following wash procedure is according to Agilent CHIP on CHIP instruction).

(20) Wash in Wash Buffer 1 for 5min at room temperature (stir with magnetic stir bar and shield the light).

(21) Wash in Wash Buffer 2 (Agilent: 5188-5222) for 1 sec at room temperature (stir with magnetic stir bar and shield the light).

(22) Wash in Wash Buffer 2 for 5min at room temperature (stir with magnetic stir bar and shield the light).

(23) Gradually stop stirring and take out the microarray slowly and absorb the wash buffer with paper towel.

(24) Dry with blowing N₂.

(25) Scan with scanner quickly.