Random Amplification of DNAs for Microarray (ChIP- chip)

Adapted from Bohlander et al Genomics 13 (1992) and modified according to DeRisi and Rando labs

NOTE 1: The goal of this procedure is to randomly amplify any given sample of DNA which as much representation as possible. It is not a "linear" method, but is useful to compare relative enrichment between two samples. This protocol has been used successfully to amplify genomic representations of less than 10ng of DNA.

NOTE 2: The protocol consists of three sets of enzymatic reactions. In Round A, Sequenase is used to extend randomly annealed primers (Primer A) to generate templates for subsequent PCR. During Round B and C, the specific primer B is used to amplify the templates previously generated. Finally, Klenow reaction consists of additional random-primed DNA synthesis to directly incorporate Cy-coupled dCTP.

Caution: Wear gloves and be careful of contamination as ANY DNA can be amplified by this protocol. Filter tips are recommended at least for Round A!!! *Always* run a control sample with water only (no DNA template) to make sure the reagents are not contaminated with DNA.

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Materials

Round A

QIAquick PCR purification kit
Sequenase (13 units/µl) US Biochemical cat# 70775
5X Sequenase Buffer (200 mM Tris pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl)
Sequenase Dilution Buffer (10 mM Tris pH 7.5, 5 mM DTT, 0.1 mM EDTA)
10 mM dNTP mix
10 mg/ml BSA
0.1 M DTT
80 µM Primer A: GTT TCC CAG TCA CGA TCN NNN NNN NN

Round B and C

10X PCR Buffer (500 mM KCl, 200 mM Tris pH 8.4)
50 mM $MgCl_2$
10 mM dNTP mix
Platinum Taq polymerase
100 µM Primer B: GTT TCC CAG TCA CGA TC
Qiagen MinElute PCR purification kit

Random-primed DNA synthesis with Cy-dCTP by klenow (Direct labeling)

2.5X random primer solution (BioPrime DNA labeling System, Invitrogen 18094-011)
125 mM Tris pH6.8
12.5 mM $MgCl_2$
25 mM 2-mercaptoethanol
750 µg/ml random octamers

10X dNTP mix
1.2 mM dGTP
1.2 mM dATP
1.2 mM dTTP
0.6 mM dCTP
CyDye5-dCTP (Amersham PA55021); Blue (turned into Red signal when scanned) – for INPUT
CyDye3-dCTP (Amersham PA53021); Red (turned into Green signal when scanned) – for ChIPed
Klenow (40 U/µl)
0.5 M EDTA pH8.0

Protocol

For a ChIP done on chromatin from 30 ml of cells (800 µl of chromatin solution), dissolve the decrosslinked DNA in 40 µl of ddH_2O and use all for labeling.

For total DNA INPUT, if 50 µl of chromatin solution was decrosslinked and dissolved in 50 µl of ddH_2O, dilute 1/50 with ddH_2O and use 40 µl for labeling.

1. Round A Reactions

Denature template DNA/primer annealing
As little as 10 ng of DNA can be effectively amplified by this protocol.

Chromatin DNA + ddH_2O	40 µl
5X Sequenase Buffer	12 µl
80 µM Primer A	2 µl (GTT TCC CAG TCA CGA TCN NNN NNN NN)

program name: **ROUND-A**
[94°C for 4 min, 10°C hold for 5 min, Ramp to 37°C **over 8 min**, 37°C hold for 8 min] X 2

Heat 4 min at 94°C
Snap freeze samples
(Once cycler temperature reaches 10°C, place snap frozen samples back in cycler).
10°C hold for 5 min
Add Reaction Mixture to sample:

10 mg/ml BSA	0.5 µl
0.1 M DTT	3.0 µl
10 mM dNTP	2.0 µl (Final 333 µM)
1/5 diluted Sequenase (1.3U/µl)	1.0 µl

Ramp from 10 °C to 37 °C over 8 min.
Hold at 37 °C for 8 min; rapid ramp to 94 °C and hold for 4 min.

Snap freeze samples
(Once cycler temperature reaches 10°C, place snap frozen samples back in cycler).

Hold for 5 min at 10 °C while adding 1.0 µl of 1/5 diluted Sequenase.
Ramp from 10 °C to 37 °C over 8 min.
Hold at 37°C for 8 min

Purify Round A products through Qiagen QIAquick PCR purification kit, wash the column twice, and elute with 85 µl of 5 mM Tris, pH8.5 (dilute the provided Elute Buffer by 1/2 fold).

2. Round B PCR

Round A DNA from above	83 µl
10X PCR Buffer	10 µl
50 mM MgCl2	3 µl
10 mM dNTP mix	2 µl (Final 200 µM)
100 µM Primer B (GTT TCC CAG TCA CGA TC)	1 µl
Platinum Taq polymerase	1 µl

program name: **ROUND-B** (uses 17 cycles depending on the amount of starting material.)
94°C 4 min

94°C 30 sec
40°C 30 sec
50°C 30 sec
72°C 1 min

4°C 20 min

Run 5 µl on 1 % agarose gel. A "smear" of DNA should be present between 300 bp –1 kb. It may be necessary to remove aliquots every 2 cycles to check the amplification in order to optimize the number of cycles. It is best to use the minimal number of cycles that generates a visible smear. Make sure there is no DNA in the negative control lane!

3. Round C PCR

Round B DNA from above	20 µl
10X PCR Buffer	10 µl
50 mM MgCl2	3 µl
10 mM dNTP mix	2 µl (Final 200 µM)
100 µM Primer B (GTT TCC CAG TCA CGA TC)	1 µl
ddH_2O	63 µl
Platinum Taq polymerase	1 µl

program name: **ROUND-C** (uses 16 cycles.)
94°C 4 min

94°C 30 sec

40°C 30 sec
50°C 30 sec
72°C 1 min

4°C 20 min

PCR product should be 20-25 ng/µl (total 2.0-2.5 µg/rxn).
Run 5 µl on 1 % agarose gel to check a successful amplification.

Purify with Qiagen MinElute; wash twice, and elute 12 µl of Elution Buffer (10 mM Tris pH8.5) twice (total ~21 µl). DNA may now be stored at -20°C.

4. Random-primed DNA synthesis with Cy-dCTP by klenow (Direct labeling)

Add 20 µl of random primer mix to 21 µl of Round C elute (usually ~ 2 µg).
Boil for 5 min and rapidly chill on ice for 5 min. Flash spindown.

Add the following;

10X dNTP mix	5 µl
Cy-coupled dCTP	3 µl
Klenow (40 U/µl)	1-1.2 µl

Incubate at 37°C for 2-3 hr. Add 5 µl of 0.5 M EDTA to terminate the labeling reaction.

Prepare Microcon 30 column and add 450 µl of ddH_2O
Mix the Cy5 and Cy3-labeled DNAs together and add to Microcon 30 column.
Centrifuge for 10 min at 10,000 rpm.
Remove the filtrate and add 550 µl of ddH_2O and 5 µl of yeast tRNA (20 µg/µl).
Centrifuge again for 10 min at 10,000 rpm.

Invert the Microcon column and recover the samples by spinning at 10,000 rpm for 1 min.

5. Hybridization with microarray slides

	Small Array	Large Array
add ddH_2O up to	24 µl	40 µl
20X SSC	5.1 µl	8.5 µl
10% SDS	0.9 µl	1.5 µl (be careful not to add extra carryover of SDS)

Boil for 3 min and Store at RT for 10 min in the dark.
Prepare dust-free coverslip with N_2 spray ; 24 X 40 mm (small array), 24 X 60 mm (large array).
Add 50 µl of 3X SSC to each well of hybridization chambers.

Place the array in the hybridization chamber (array # side down) and put the labeled DNAs on it.
Use sharp pincep or forcep to put down the coverslip. Try to minimize the air bubble captured inside.

Always handle the arrays flat!!!
Hybridize at 55°C for 3-4 hr (water bath).

6. Washing and scanning with GenePix 4000B

Warm up the scanner first (fire up the software).
Place the chamber top on a flat styroform and wipe out water with paper towels.
Disassemble the chamber.
Wash the array with 1X SSC/0.03% SDS for 3 min.
(before soaking the slides, remove any bubble in the buffer first).

Wash with 0.2X SSC for 3 min (when transfer from previous wash, use pincep and try to minimize SDS carryover. Also use kimwipes if necessary).

Wash with 0.05X SSC for 3 min (use slide carrier box for transfer from previous wash).

Centrifuge for 3 min at 1,000 rpm for spin-dry.
Put the array slides into a light-tight box.
Scan the array and analyze.
(try to minimize the number of scan because it quenches the siginal.)

For Ollie's Ch III array, the followings are optimal scanning parameters (if signals are bright);

	635 (Cy5)	532 (Cy3)
PMT value	600-700	590-640
Power (%)	100	100

Pixel size	5 µm
Lines to average	2
Focus position	0 µm