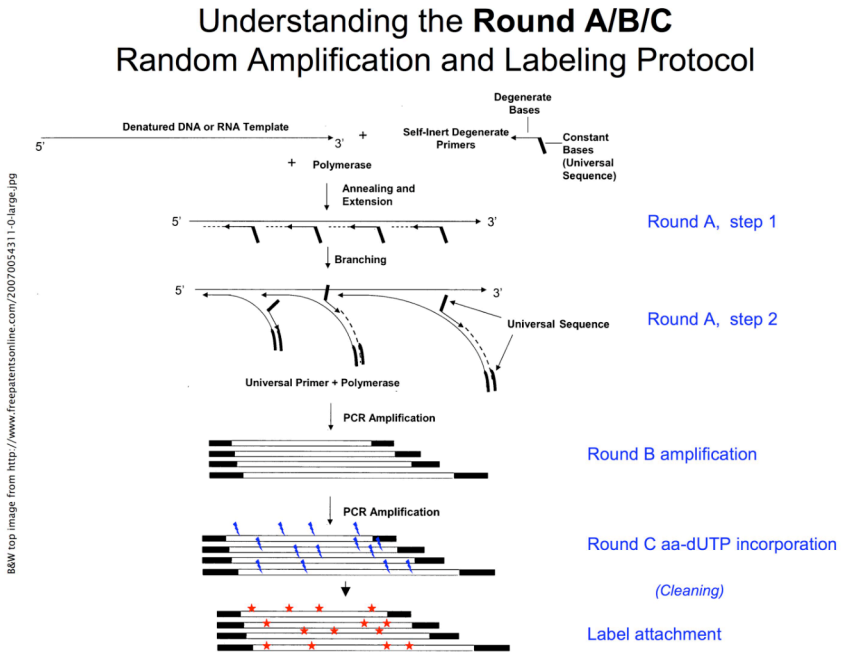
By Virginia Rich, graduate student in the DeLong Lab, vrich@mit.edu
Last modified 4/28/08

Amplification and Labeling of DNA for Microarray Hybridization, using the "Round A/B/C" Random DNA Amplification Protocol

Goal: To amplify and label DNA prior to hybridization to a microarray, in a relatively random way. This protocol does not give linear amplification, but as DeRisi says it "is useful to compare relative enrichment between two samples." DeRisi reports that it has been successful in their hands for amplifying less than 1ng of genomic DNA. I have obtained results from as little as several hundred picograms of environmental DNA but a safer lower limit starting amount of DNA seems to be ~6ng per slide hyb (see caveats below).

Protocol History: I adapted this protocol from that used by Joseph DeRisi's Lab at U.C. San Francisco, and theirs was adapted from Bohlander et al. *Genomics* 13 (1992).

Overview: There are three stages of this protocol. In Round A, the Sequenase polymerase extends random primers with specific ends (Primer A) that have annealed to the template DNA. In Round B, conventional PCR amplifies the templates from Round A, using the specific primer (Primer B) which matches the 3' end of Primer A. In Round C, Primer B is used again to mediate rounds of conventional PCR during which modified nucleotides are incorporated for labeling. These modified nucleotides are typically either amino-allyl-dUTP, for indirect labeling, or nucleotides that are directly coupled to Cy dyes. I use the aa-dUTP indirect labeling so that is what is described here. There is less discrimination by the polymerase against the smaller aa-dUTPs than against large, bulky Cy-dNTPs.



Precautions: This is a random-amplification protocol, which means that *ANY* DNA can be amplified. Therefore, use filter tips, wear gloves, and UV-sterilize your tubes along the way. Also, run negative control reactions. Also, because it's a random-primed multi-round exponential amplification, we might worry about stochastic skewing of the relative abundance of different organisms during amplification. The protocol partly accounts for this by subsampling each amplification as the template for the next round (shown to be beneficial to PCR evenness generally in Thompson *et al.*, 2004). In addition, I choose to **run triplicate amplification reactions and pool them prior to labeling**. Pooling multiple reactions has also been shown to decrease random biases introduced early during amplification (Thompson *et al.*, 2004).

Materials

Item	*Supplier*	*Item #*
General:		
Nuclease-Free Water	Applied Biosystems/Ambion	#AM9937
Positive control DNA		
In my case, *Halobacterium*	ATCC	#700922D
Round A		
Sequenase (13 units/μl)	US Biochemical	#70775
5X Sequenase Buffer	included	
Sequenase Dilution Buffer	included	

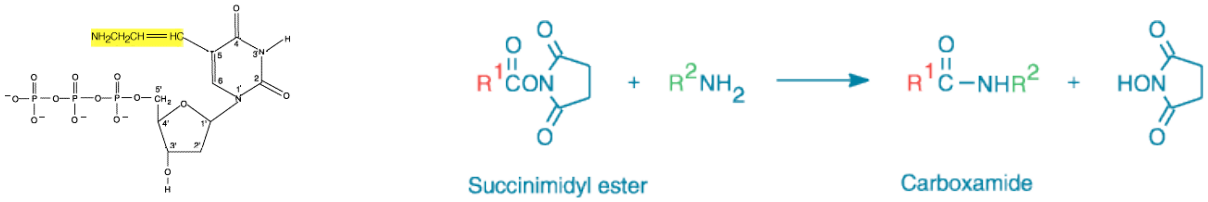
"Sequenase Version 2.0 DNA polymerase is a genetically engineered form of T7 DNA polymerase which retains extraordinary polymerase activity with virtually no 3'->5' exonuclease activity. It is highly processive, able to effectively incorporate nucleotide analogs for sequencing (dideoxy NTPs, -thio dATP, dITP, 7-deaza-dGTP, etc.) and is not easily impeded by template secondary structure."

"A" dNTPs = 3 mM each nucleotide
500 μg/ml BSA
0.1 M DTT
40 pmol/μl Primer A: e.g. Proligo N/A
5' - GTT TCC CAG TCA CGA TCN NNN NNN NN - 3'

Round B
10X Mg-minus PCR Buffer to match the Taq
(500 mM KCl, 100 mM Tris pH 8.3)
25 or 50 mM MgCl2
"B" dNTPs = 25 mM each nucleotide
5 U/μl Taq polymerase e.g. Invitrogen
100 pmol/μl Primer B: e.g. Proligo N/A
5' - GTT TCC CAG TCA CGA TC - 3'

Round C
Same as Round B except use modified "C" dNTP mix:
Their recommended recipe is:
25 mM each dATP. dCTP and dGTP
10 mM dTTP
15 mM aminoallyl-dUTP (or Cy-dUTP) Ambion #AM8439
However, they suggest that the ratio of aa-dUTP to dTTP can be altered/optimized. My optimized recipe is:
22.5 mM each dATP. dCTP and dGTP
9 mM dTTP
11.75 mM aminoallyl-dUTP
For 100 μl this corresponds to:
22.5 μl 100mM dATP, dCTP, and dGTP
9 μl 100mM dTTP
23.5 μl 50mM aa-dUTP

aa-dUTP structure (for L-A ☺), and the general amine-ester reaction employed for dye coupling:



Protocol

Round A: Denature template DNA, anneal primers, and extend.
Time: At least 1 hour 20 minutes, increasing significantly based on number of reactions.

Round A, Step 1: First strand synthesis.
Each reaction receives:

Ingredient	*Volume*
Template DNA	7 µl
(e.g. 6 µl template DNA and 1µl positive control DNA)	
5X Sequenase Buffer	2 µl
Primer A (40 pmol/µl)	1 µl
Total Volume = 10 µl	

To standardize things I prepare a master mix of 5X Sequenase buffer and Primer A, and then dispense it into my tubes – either 0.2ml PCR tubes or a PCR plate. For triplicate reactions, I dispense 3X of this master mix into the wells, add 3X of my DNA, mix, and then aliquot into three separate tubes, or three separate rows if using a PCR plate. This works well.

Use "vr-a" cycling protocol on "Goldie" thermal cycler**:
Heat 2 min at 94 ˚C
Rapid cool to 10 ˚C and hold 5 min at 10 ˚C.

*** I use this thermal cycle because I have programmed it to have approximately the correct ramp time for later steps. Other thermal cyclers have different ramping speeds and so will need to be programmed accordingly.*

With program paused at 10 ˚C and the tubes in the thermal cycler, add 5.05 µl **Reaction Mixture** to each reaction (having assembled reaction mixture in UV-hood):

Ingredient	*Volume*
5X Sequenase Buffer	1 µl
"A" dNTPs (3mM)	1.5 µl
0.1 M DTT	0.75 µl
500 µg/ml BSA	1.5 µl
Sequenase (13U/µl)	0.3 µl
Total Volume = 5.05 µl	

Again, I make a master mix of this reaction mixture, in the UV-hood, and then dispense it at the thermal cycler into each tube or well.

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 2 min.
Rapid ramp to 10˚C and hold for 5 min

Round A, Step 2: Second strand synthesis
Pause at 10˚C while adding 1.2 µl of diluted Sequenase (1:4 dilution in Sequenase Dilution Buffer).

Ramp from 10 ˚C to 37 ˚C over 8 min.
Hold at 37˚C for 8 min.
END

In PCR hood, dilute samples with Ambion Water to final volume = 60 µl (should be 60 – 10 – 5.05 – 1.2 = 43.75ul).

Round B: PCR amplification.
Time: ~2-4 hours, depending on # of cycles run.

Mix in a 0.2ml PCR tube, in the UV-hood:

Ingredient	*Volume*
Round A Template	6 µl
50mM MgCl2	4 µl
10X Mg-minus PCR Buffer	10 µl
"B" dNTPs (25mM)	1 µl
Primer B (100pmol/µl)	1 µl
Taq	1µl
Ambion Water	77µl
	Total Volume = 100 µl

Use "vr-b" cycling protocol on "Goldie" thermal cycler:
30 sec at 94 ˚C
30 sec at 40 ˚C
30 sec at 50 ˚C
2 min at 72 ˚C

Run 15-35 cycles, depending on the amount of starting material. I typically use 20 cycles.

If you run 5 µl of each reaction product on a 1% agarose gel, you should see a smear of DNA between 500bp -1kb. To minimize the number of cycles you run, the first time you're working with a new type of template they recommend removing aliquots of your reaction (of which you have extra to spare, don't worry) every 2 cycles or so and checking them on a gel – you want to use the minimum number of cycles that produces a visible smear of product DNA, and that still keeps the negative control lanes empty.

Round C: Incorporation of aa-dUTP.
Time: ~1-2 hours, depending on # of cycles run.

They recommend using 10-15 µl of Round B to seed the Round C reaction. I use 10 µl.

Ingredient	*Volume*
Round B Template	10 µl
50mM MgCl2	4 µl
10X PCR Buffer	10 µl
"C" dNTP mix	1 µl
Primer B (100pmol/µl)	1 µl
Taq	1 µl
Water	73 µl
	Total = 100ul

Use "vr-c" cycling protocol on "Goldie" thermal cycler:
30 sec at 94 ˚C
30 sec at 40 ˚C
30 sec at 50 ˚C
2 min at 72 ˚C

10-25 cycles can be run, I typically run 10 cycles.

Clean-up I:

Salts and Tris interfere with dye coupling, so before proceeding you must clean up the reactions. They recommend using a Microcon size-exclusion column to do this.

Add 400 μl water to the sample in a Microcon 30
Spin 14,000xg until liquid mostly drained. Empty collection tube.
Wash 1X with 500 μl Ambion water.
Concentrate to ~9.3 μl in Ambion water: 8 μl will be used for the labeling reaction, and ~1 μl will be used to Nanodrop the sample. I record the volume I was actually able to concentrate the sample to (it's tricky, and I can't usually get to 9.3 exactly) so that I can calculate my amplification efficiency if I want to, and also understand the comparability of my samples. Obviously, one wants the volumes to be as close as possible to one another between samples to permit the most comparability.

Also, I combine my triplicate reactions at this stage, pre-labeling. You can combine triplicates prior to Microconning but beware that if you pool the negative controls before you clean them they may be VERY slow to drain.

If doing lots of samples, instead of using Microcons, I use an ExcelaPure 96-well size-exclusion-column plate with the vacuum manifold. I run it at 10"Hg so as not to lose DNA <300bp. Note that this size exclusion cutoff is a little bigger than the Microcon-30's. SO, for any experiment or for experimental series you'd like to be able to compare, it would be advisable to consistently use one or the other clean-up method.

Wash 1 x 300 μl of Ambion water
Resuspend in ~30 μl Ambion water, transfer to a v-bottom 96 or 384-well plate.
Use the vacuum centrifuge with the plate rotor to dry down the DNA. Use e.g. the automatic spin with 2 hours vacuum spin, 1 hour at 45 deg. C. Then resuspend your DNAs directly in 0.1M NaHCO3 (allowing to sit at e.g. 60 deg C for 10", then vortex gently and spin).

Labeling:

8 μl aa-DNA
2 μl 0.5M NaHCO3 (0.1M final concentration in the DNA mixture)
mix
OR 10 μl aa-DNA resuspended in 0.1M NaHCO3
5 μl Cy dye (33 μg in DMSO)
mix
incubate at room temperature in the dark for 1 hour.

Co-spot complement: If you are using the co-spot complement as well, you will have done a single separate labeling reaction of that, linked to Cy5. I've found that using ~1pmol of the co-spot complement oligo per array hybridization works fine. In my case, the co-spot complement that performed the best and that I ended up using was the "alien" complement oligo from Urisman *et al.*, 2005.

Quenching: If using the co-spot complement, you'll combine the differently-labeled DNAs at the hybridization stage. You wouldn't want any residual uncoupled dyes to cross-label the wrong DNA. Although rinsing with TE will quench the labeling reaction, and should remove uncoupled dyes, for best practices you should ALSO use the traditional chemical quenching protocol step of adding 2 μl of 4M hydroxylamine to each reaction, mixing, and allowing them to sit in the dark an additional 15 minutes.

Clean-up II:

Now you want to remove unincorporated dye molecules. Use the single-column or 96-well size exclusion column plates, as before. Now, however, wash with TE. The TE helps inactivate the dye conjugation process.

Add 480 μl (or 280 μl if using Excela-Pure plates) to your samples.
Transfer to the columns.
Spin.
Wash columns 2x 500 μl TE (or 2x 300 μl if using Excela-Pure).
Concentrate to ~19 μl in TE, or more if you're doing triplicate slides.

Note: do not use the Excela-Pure plate to clean the co-spot complement. This oligo is smaller than the cutoff of the Excela-pure columns.