Rolling Circle Amplification

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SUMMARY

This protocol outlines the procedure of amplifying DNA template for sequencing.

MATERIALS & REAGENTS

Materials/Reagents/Equipment	*Vendor*	*Catalog Number*
DISPOSABLES		
96 well PCR plates	ISC Bioexpress	T-3082-1
0.1 µL – 10µ L Natural tips (tip one)	USA Scientific	1111-3200
Clear Seal	Marsh BioProducts	AB-0812
96 well U Bottom plates, Sterile	ISC Bioexpress	T-3016-2
Termalseal Tape	ISC Bioexpress	T-2417-5
REAGENTS		
TempliPhi™ 1000 Reaction Kit TempliPhi ™ Denature Buffer TempliPhi ™ PreMix	Amersham Pharamacia Biotechs, Inc.	25-64001
TE Buffer	TekNova	T0223
STOCK SOLUTIONS		
Water, HPLC grade	Aldrich	7732-18-5
EQUIPMENT		
MJ Thermal Cycler	MJ Research	
Hydra	Robbins Scientific	
Plate Reader		
Incubator set at 30℃		
Hi-Gro	GeneMachines	
Thermal Sealer	Combi	

PROCEDURE

Preparation

1. First thing in the morning remove the innoculated *E.coli* plates out of the Hi-Gro and place it in the 4°C refrigerator. This will allow the *E.coli* plate to slow its growth, thus minimizing dead cell density.

2. Wash the Hydra by setting it on FILE 1. Make sure the WV is full and WASH 15 in the LED display on the Hydra. To check this scroll through file 1 using the red set/reset button. Wash with Coulter Clenz for 1 whole cycle, and DI water for 2 whole cycles, a total of 45 full washes. Please change the Coulter Clenz solution once a week.
3. Before beginning RCA setup, place the *E.coli* plate in the Hi-Gro for 10 minutes at 410 rpm. This will ensure that the samples are given ample time for the *E-coli cells* to become homogeneous.

Before Beginning Set-up

1. Remove the needed aliquoted plates filled with 3 uL of TempliPhi™ denature buffer in each well from the –20°C freezer C. There are 96 well plates already filled with 3 uL of TempliPhi™ denature buffer located in Freezer C on the 3rd shelf, please make some more aliquoted plates when necessary. **NOTE: The denature buffer can be thawed at room temperature.**
2. Remove the TempliPhi™ premix from the –80°C freezer. The premix can be found in the blue or green 96 well plate holders labeled RCA Reaction Mix located in the bottom shelf. The premix is aliquoted enough for 4 plates into 0.2 mL 12-tube strips. Please aliquot more when necessary. **NOTE: The premix has to be kept cold AT ALL TIMES. It can be thawed on a cooling block or ice, but make sure that the block is cold and that the enzyme does not reach 4°C. If the enzyme reaches 4°C it will become active and start amplifying primers.**
3. Place the appropriate barcode label on front of the plate.
    a. The barcode is retrieved from the CGF website.
    b. Select **CGF Staff Login** and login using username and password.
    c. Select **Sequencing Jobs in Progress.**
    d. Select the appropriate job from the list.
    e. Enter in the Library name.
    f. Select the plates that are going to be RCA'd and press the Select button. This should print out the appropriate barcodes. Place these barcodes on the front side of the plate.

Beginning Setup:Denaturation Step

1. Grab the *E-coli* plates out of the Hi-Gro, but make sure it has been in there for at least 10 minutes to ensure proper mixing.
2. Grab the desired 96 well *E-coli* plate and place it on the dispensing platform. Make sure to work slowly and accurately. Set the Hydra at FILE 2
3. Push the FILL button to pick up 3.0 uL of *E-coli*. Push the red Aspirate/Dispense button to dispense back into the plate. Repeat this for an additional time for a total of two times.
4. Then remove the *E-coli* plate from the dispensing platform, and place the appropriately labeled *aliquoted plate filled with 3 uL of TempliPhi™ denature buffer* on the dispensing platform.
5. Push the red Aspirate/Dispense button, it should dispense 0.5uL of the *E-coli* into the plate. Note: Grab the plate with two hands to ensure that 0.5 uL is dispensed properly into the plate.
6. Seal the plate using TermalSeal Tape and make sure to use a roller, rolling over it a couple of times.
7. **Wash steps for Hydra.** Grab the wash reservoir that is labeled WASTE. Pour 2% Bleach in until it's almost full and place it on the dispensing platform. Leave it on FILE 4 and push the EMPTY button. Remove the waste reservoir and place the reservoir filled with water on the

platform. Push the WASH button. When the was is done, throw out the waste water in the waste bucket and repeat the wash step 2 more times for a total of 9 washes.

1. Repeat steps 1-8 for the other plates. Spin down the plates in the centrifuge for less than a minute (This can be done in groups of 4).
2. Place the plates in the MJ Thermal Cycler.
3. Run "RCA-DENA" program (95°C for 5 minutes, 4°C ∞).
   Note: Make sure that the wells of the plate are tightly in contact with the block surface of the thermal cycler.

Amplification

1. After the denaturation step, make sure the temperature is in between 25-30°C for the next reactions.
2. Add 3.0μL TempliPhi™ premix to the denatured plate and make sure to change tips in between every row/column if the tips touch the plate. **MAKE SURE THE PREMIX IS KEPT ON THE COLD BLOCK AT ALL TIMES. ALSO WHEN ADDING THE PREMIX TO THE DENATURED PLATE, MAKE SURE THAT THE 96 WELL PLATE IS KEPT ON A COLD BLOCK AS WELL.**
3. Seal the plate with Clear Seal by using the Thermal Sealer. Press down for 10 seconds.
4. Make sure the sealing film is tightly sealed so the reaction will not evaporate in the wells.
5. Place it in the centrifuge for a quick spin (1000 RPM for 10 seconds).
6. Place the plate in the Styrofoam box labeled RCA REACTIONS INSIDE. Place the Styrofoam box in the incubator located in the Q-bot room. Make sure the incubator is set at ~27.8 degrees and leave it overnight.