Sequencing Reaction Setup

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SUMMARY

This protocol outlines the procedure of setting up sequencing reactions.

MATERIALS & REAGENTS

Materials/Reagents/Equipment	*Vendor*	*Catalog Number*
DISPOSABLES		
Half Skirted 96 well PCR plates or Regular 384 well plates	ISC Bioexpress	T-3060-1
0.1 uL – 10 uL Natural tips (tip one)	USA Scientific	1111-3200
Clear Seal	Marsh Products	AB-0812
Seal-Rite™ 2.0 mL Natural Microcenrifuge Tubes	USA Scientific	1620-2700
Seal-Rite™ Locking 1.5 mL Natural Microcentrifuge Tubes	USA Scientific	1615-5100
Solution Basin, Reservoir	ISC Bioexpress	B08122
Full Skirted 96 well PCR plates	ISC Bioexpress	T-3082-1
REAGENTS		
3730-V3.1 BigDye Terminator	Applied Biosystems	4337457
DNA Sequencing Buffer	Tek Nova	D1301
Primers	Invitrogen Life Technologies	
STOCK SOLUTIONS		
Water, HPLC	Sigma Alrich	270733-6X1L
EQUIPMENT		
Thermal Sealer	Combi	
MJ Thermal Cycler	MJ Research	
Mini Vortexer	VWR Scientific Products	
Centrifuge	Beckman Coulter	
Hydra	Robbins Scientific	

PROCEDURE

Preparation

1. After the RCA amplified sample has sat overnight, the enzyme is inactivated, and the quantitation of the DNA looks good, then it is ready for sequencing reaction setup
2. You will need a bucket of ice to ensure that all the ingredients for the sequencing are kept cold. It is very important that all the ingredients are kept on ice at all times during the sequencing reaction setup.
3. Remove the needed amount of Big Dye 3.1 from the -20 *°C* Freezer C located on the top shelf contained in 2.0 mL natural microcentrifuge tubes. There are aliquots of 1 mL of Big Dye found in the blue cooler labeled Big Dye Version 3.1. Place it on ice to let it thaw.
4. Remove the needed primers from the -20 *°C* Freezer C located on the third side shelf in the blue cooler, these are aliquoted in smaller volumes. The primers that contain a larger volume can be found in the white cooler. Use these to aliquot into smaller volumes.
5. Remove the DNA Sequencing Buffer from the 4 *°C* refrigerator located on the top shelf labeled DNA SEQUENCING BUFFER contained in a red 50 mL conical tube. If more is needed the bottle is located on the first side shelf of the refrigerator. Place both the primers and buffer on ice.
6. Label the 96 well PCR plates with the appropriate barcode label in the 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. Check the appropriate boxes in the RCA PLATES section.
    f. Click on the SELECT button and the appropriate barcodes should be printed out. Place these barcodes on the front side of the plate.

Sequencing Master Mix Addition (1/8 dilution)

1. Once the ingredients are thawed, figure out how much of each ingredient is going to be used.

MASTER MIX

	1 plate x 96	4 plates x 96	8 plates x 96	16 plates x 96
Sequencing Buffer	50 uL	200 uL	400 uL	800 uL
ddH20, HPLC grade	195 uL	780 uL	1560 uL	3120 uL
Big Dye V. 3.1	50 uL	200 uL	400 uL	800 uL
Primer (100 pmole/uL)	5 uL	20 uL	40 uL	80 uL

2. Add the ingredients and mix the solution in a 1.5 mL natural microcentrifuge tube, 2.0 mL natural microcentrifuge tube, or 15 mL microcentrifuge tube, whichever best fits all the mixture.
3. Vortex the mixture on the mini-vortexer for a couple of seconds to ensure proper mixture.
4. Grab a reservoir and place it on ice. Pour the mixture in the reservoir and add 2.5 uL to each label 96 well plate. Note: Make sure the labeled plate is on a cold block. Always keep the master mix cold. Work slowly and carefully making sure every well gets 2.5 uL of master mix.
5. Grab the desired amplified RCA plate and vortex it for a couple of seconds carefully. Add 2.5 uL of amplified RCA product to each well using the hydra. Using the hydra will ensure uniformity of the dispensed product.
6. Set the Hydra to FILE 7 with the DV at 2.5 uL and the FV at 3.0 uL.
7. Place the Hydra in ready mode by pushing the set/reset button until the LED display reads D 0.0.

8. Press the FILL button and then place the appropriately labeled half-skirted plate on the dispensing platform.
9. Press the red DISPENSE/ASPIRATE button. This will dispense 2.5 uL of RCA product into the plate. Note: Empty into a basin filled with water and wash as usual.
10. Seal the plate with Clear Seal using the Thermal Sealer. Press down for 2 seconds and let go. If you press down too long, the plate will melt and it will be harder to do ethanol precipitation later on.
11. Place the plate in the centrifuge and spin it until it reaches 2000 RPM then stop the spin.
12. Place the plate in the thermal cycler under the program name- BD-SQN.

BD-SQN THERMAL CYCLER CONDITIONS

95° C 30 seconds
96° C 20 seconds
50° C 15 seconds
60° C 4 minutes
GoTo 2, 40 X
4° C Forever

Using the Hydra to Setup Sequencing Reaction

1. Use the Hydra to add sequencing master mix for 8 plates or more. After adding the needed ingredients and mixing them, aliquot it into a full skirted 96 well plate. Spin it down for a couple of seconds.
2. Set the Hydra program to FILE 7 if your adding the master mix into half skirted 96 well plates. Set the Hydra program to FILE 8 if your adding the master mix into 384 well plates. Set DV to 2.5 and FV to half of what is in the aliquoted plate. For example, I am going to add master mix to 16 plates (2.5 x 16 plates= 40), so I take half of this and set the FV to 20 plus always add an extra 1 uL or 2. Thus, the FV will be set to 21. Place the Hydra in ready mode by pushing the set/reset button until the LED display reads D 0.0.
3. Place the aliquoted full skirted 96 well plate on the dispensing platform. Push the FILL button.
4. Remove the aliquoted full skirted 96 well plate and place the half skirted 96 well plate or the 384 well plate on the dispensing platform. Note: If you are using a 384 well plate you need to place it on the plate positioner and move the wheel from quadrant I to quadrant IV as you add master mix to each quadrant. Press the red ASPIRATE/DISPENSE button.
5. Repeat until all the plates are finished. Remember to leave the plates on ice after dispensing the master mix. It is important that they are kept cool.
6. Wash twice using fresh water both times by pressing the WASH button.
7. When adding amplified RCA product set the plate containing the RCA product on the dispensing platform and set FV at 3.0 and DV at 2.5. Press the FILL button.
8. Remove the RCA plate and place the master mix plate filled with 2.5 uL of master mix in each well on the dispensing platform. Press the ASPIRATE/DISPENSE button. Empty the excess in the waste reservoir by pressing the EMPTY button.
9. Wash twice using fresh water both times by pressing the WASH button.
10. Seal the plate with Clear Seal by using the Thermal Sealer. Spin down for a few seconds and place it in the thermal cycler under the program BD-SQN.