NimbleGen DNA Methylation Microarrays

Sample Labeling Instructions

Outline

This protocol describes the process for labeling samples before hybridization to NimbleGen DNA Methylation arrays. It is intended for use by researchers who want to deplete existing stock of reagents before transitioning to NimbleGen Dual-Color DNA Labeling Kits.

Use this protocol as a replacement for only Chapter 3, Sample Labeling, of the *NimbleGen Arrays User's Guide: DNA Methylation Analysis*. After you complete sample labeling, follow the instructions in the other chapters of the user's guide for complete processing of DNA Methylation arrays.

Protocol Information & Safety

Refer to the *NimbleGen Array's User's Guide: DNA Methylation Analysis* for protocol information and safety considerations.

Required Reagents/Consumables

Component	Supplier	Package Size	Item Number
β-Mercaptoethanol	Sigma Aldrich	25ml	M3148
0.5M EDTA	Sigma Aldrich	100ml	E7889
100mM dNTPs	Invitrogen	4 x 25µmol	10297-018
1M $MgCl_2$	Sigma Aldrich	100ml	M1028
1M Tris HCl, pH7.4	Sigma Aldrich	1 liter	T2663
5' Cy3- and Cy5-labeled Random Nonamers (9mer "Wobble")	TriLink Biotechnologies	50 O.D.	N46-0010-50
		200 O.D.	N46-0010-200
5M NaCl	Sigma Aldrich	250ml	71386
Absolute Ethanol	Sigma Aldrich	500ml	E702-3
Isopropanol	Sigma Aldrich	500ml	I-9516
Klenow Fragment 3'->5' exo-	NEB	50U/µl	M0212M
Water: reagent grade, ACS, nonsterile, type 1	VWR	2.5 gallon	RC915025

Sample Labeling

Pairs of samples intended for hybridization to the same array should be labeled in parallel using Cy3- and Cy5-Random Nonamers from the same lot. Roche NimbleGen recommends labeling experimental (IP) samples with Cy5 and control (input) samples with Cy3.

1. Refer to Chapter 2, Sample Preparation & QC, of the *NimbleGen User's Guide: DNA Methylation Analysis* for the sample amount required for the array format.

2. Prepare the following three solutions:

10X TE (100mM Tris HCl, 10mM EDTA)	All Array Formats	Notes
1M Tris HCl, pH 7.4	1.5ml	Mix and store at room temperature.
0.5M EDTA	0.3ml	
VWR water	13.2ml	
Total	**15ml**	
50X dNTP Mix	**All Array Formats**	**Notes**
VWR water	250µl	Aliquot 50X dNTP mix into single-use amounts and freeze. Avoid freeze-thaw cycles, which can result in diminished yields. When in use, keep dNTPs on ice at all times.
10X TE	50µl	
100mM dATP	50µl	
100mM dGTP	50µl	
100mM dTTP	50µl	
100mM dCTP	50µl	
Total	**500µl**	
Random Primer Buffer	**All Array Formats**	**Notes**
VWR water	8.6ml	Prepare fresh buffer each time primers are resuspended. 42µl of Random Primer Buffer is needed per O.D. of 9mer primer.
1M Tris HCl, pH 7.4	1.25ml	
1M $MgCl_2$	125µl	
β-Mercaptoethanol	17.5µl	
Total	**~10ml**	

3. Dilute Cy3 and Cy5 dye-labeled 9mers to 1 O.D./42µl Random Primer Buffer. Aliquot to 40µl individual reaction volumes in 0.2ml thin-walled PCR tubes and store at -20°C, protected from light.

4. Assemble the experimental (IP) and control (input) samples in separate 0.2ml thin-walled PCR tubes.

Component	385K and 4x72K Arrays		2.1M Arrays	
	Experimental (IP) Sample	Control (Input) Sample	Experimental (IP) Sample	Control (Input) Sample
Sample	1µg	1µg	1µg (in each of 3 tubes)*	1µg (in each of 3 tubes)*
Cy3-labeled 9mers		40µl		40µl (per tube)
Cy5-labeled 9mers	40µl		40µl (per tube)	
VWR water	To volume (80µl)	To volume (80µl)	To volume (80µl) (per tube)	To volume (80µl) (per tube)
Total	**80µl**	**80µl**	**80µl (per tube)**	**80µl (per tube)**

* 2.1M arrays require three labeling reactions each of experimental (IP) and control (input) sample per slide. Therefore, for 2.1M arrays, assemble three separate 0.2ml thin-walled PCR tubes with each containing 1µg DNA for each experimental (IP) and control (input) sample. Experimental (IP) and control (input) sample pairs intended for hybridization to the same 2.1M array should be labeled in parallel.

5. Heat-denature samples in a thermocycler at 98°C for 10 minutes. Quick-chill in an ice-water bath for 2 minutes.

   Important: *Quick-chilling after denaturation is critical for high-efficiency labeling.*

6. Prepare the following dNTP/Klenow master mix for each sample prepared in step 5.

   Important*: Keep all reagents and dNTP/Klenow master mix on ice. Do not vortex after addition of Klenow.*

dNTP/Klenow Master Mix: Recipe per Sample	All Array Formats
50X dNTP mix	10µl
VWR water	8µl
Klenow (50U/µl)	2µl
Total	**20µl**

7. Add 20µl of the dNTP/Klenow master mix prepared in step 6 to each of the denatured samples prepared in step 5. Keep on ice.

Component	All Array Formats	
	Experimental (IP) Sample	Control (Input) Sample
Reaction volume from step 5	80µl	80µl
dNTP/Klenow Master Mix from step 6	20µl	20µl
Total	**100µl**	**100µl**

8. Mix well by pipetting up and down 10 times.

   Important*: Do not vortex after addition of Klenow.*

9. Quick-spin to collect contents in bottom of the tube.

10. Incubate for 2 hours at 37°C in a thermocycler with heated lid, protected from light.

11. Stop the reaction by addition of the Stop Solution (0.5M EDTA).

Component	All Array Formats	
	Experimental (IP) Sample	Control (Input) Sample
Reaction volume from step 7	100µl	100µl
Stop Solution (0.5M EDTA)	10µl	10µl
Total	**110µl**	**110µl**

12. Add 5M NaCl to each tube.

Component	All Array Formats	
	Experimental (IP) Sample	Control (Input) Sample
Reaction volume from step 11	110µl	110µl
5M NaCl	11.5µl	11.5µl
Total	**121.5µl**	**121.5µl**

13. Vortex briefly, spin, and transfer the entire contents to a 1.5ml tube containing isopropanol.

Component	All Array Formats	
	Experimental (IP) Sample	Control (Input) Sample
Reaction volume from step 12	121.5µl	121.5µl
Isopropanol	110µl	110µl
Total	**231.5µl**	**231.5µl**

Note: *Up to 3 reactions containing the same sample can be combined in a 1.5ml tube and precipitated together. If combined, be sure to scale the isopropanol volume appropriately.*

14. Vortex well. Incubate for 10 minutes at room temperature, protected from light.

15. Centrifuge at 12,000 x g for 10 minutes. Remove supernatant with a pipette. Pellet should be pink (Cy3) or blue (Cy5) depending on the dye.

16. Rinse pellet with 500µl 80% ice-cold ethanol. Dislodge pellet from tube wall by pipetting a few times.

17. Centrifuge at 12,000 x g for 2 minutes. Remove supernatant with a pipette.

18. Dry contents in a SpeedVac on low heat until dry (~5 minutes), protected from light.

19. **STOP POINT**: Proceed to step 20, or store labeled samples at -20°C (up to 1 month), protected from light.

20. Spin tubes briefly prior to opening. Rehydrate pellets in 25µl VWR water per reaction.

21. Vortex for 30 seconds and quick-spin to collect contents in bottom of the tube. Continue to vortex or let sit at room temperature, protected from light, for approximately 5 minutes or until the pellet is completely rehydrated, then vortex again and quick-spin.

22. Quantitate each sample using the following formula:

    Concentration (µg/ml) = A_{260} x 50 x Dilution Factor

Note: *The NanoDrop user's manual specifies that in the Nucleic Acid module the maximum accurate reading is 3,700ng/µl, and in the Microarray module, the maximum accurate reading is 700ng/µl. Roche NimbleGen recommends using the spectrophotometer in the Nucleic Acid module. If sample concentration exceeds these values, dilute sample appropriately and re-quantitate.*

23. Based on the concentration, calculate the volume of experimental (IP) sample and control (input) sample required for each hybridization per the following table and combine both experimental (IP) and control (input) samples in a 1.5ml tube:

Sample Requirements	385K Array	4x72K Array	2.1M Array
Experimental (IP) Sample	6µg	4µg	34µg*
Control (Input) Sample	6µg	4µg	34µg*

* If the 34µg was not obtained from the 3 labeling reactions, the hybridization can be performed with as little as 24µg.

24. Dry contents in a SpeedVac on low heat, protected from light.

25. **STOP POINT:** Proceed to Chapter 4, Hybridization & Washing, of the *NimbleGen Arrays User's Guide: DNA Methylation Analysis*, or store labeled samples at -20°C (up to 1 month), protected from light.

Technical Support

If you have questions, contact your Roche NimbleGen Account Manager or Roche Microarray Technical Support. Go to *www.nimblegen.com/arraysupport* for contact information.

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