NimbleGen Arrays User's Guide

Epigenetics Arrays – ChIP-chip and DNA Methylation

Version 1.0

For life science research only.
Not for use in diagnostic procedures.

Editions

Version 1.0, Nov 2011

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Preface

Intended Use

For life science research only. Not for use in diagnostic procedures.

ChIP-chip

Chromatin immunoprecipitation-on-chip (ChIP-chip) is a powerful tool to map target protein DNA binding sites across entire genomes or within biologically important regions such as promoters. This method is used to map chromatin structure and DNA binding sites of transcription factors and polymerases.

DNA Methylation

Methylated DNA regions are accurately mapped using a combination of affinity-based enrichment, such as Methylated DNA Immunoprecipitation (MeDIP) or the Methylated CpG Island Recovery Assay (MIRA), followed by microarray analysis.

Contact Information

Technical Support

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

Manufacturer and Distribution

Manufacturer	Roche NimbleGen, Inc. Madison, WI USA
Distribution	Roche Diagnostics GmbH Mannheim, Germany
Distribution in USA	Roche Diagnostics Corporation Indianapolis, IN USA

Conventions Used in This Guide

Symbols

Symbol	Description
	Potentially hazardous situation that, if not avoided, could result in minor or moderate injury for the operator or patient.
	Important Note. Information critical to the success of the procedure or use of the product. Failure to follow these instructions could result in compromised data.
	Information Note: Designates a note that provides additional information concerning the current topic or procedure.

Text

Conventions	Description
Numbered listing	Indicates steps in a procedure that must be performed in the order listed.
Italic type, blue	Identifies a resource in a different area of this manual or on a web site.
Italic type	Identifies the names of dialog boxes, windows, tabs, panels, views, or message boxes in the software.
Bold type	Identifies names of menus and controls (buttons, checkboxes, etc.) in the software.

Chapter 1. Before You Begin

This *User's Guide* describes the protocol for the Roche NimbleGen ChIP-chip and DNA Methylation microarray applications and array formats.

Applications

Chromatin Immunoprecipitation on ChIP (ChIP-chip)

ChIP-chip is used to map chromatin structure and DNA binding sites of transcription factors and polymerases.

Most researchers performing ChIP-chip use total input DNA as the control (input). Total input DNA is a small aliquot of the starting DNA pool that does not undergo the immunoprecipitation steps.

Example procedures for chromatin crosslinking, immunoprecipitation, and amplification (WGA) are described in the NimbleGen sample preparation protocol for ChIP-chip, available from Roche Microarray Technical Support. Refer to page 5 for contact information.

DNA Methylation

This method is used to map methylated DNA regions by combining methylated DNA immunoprecipitation with microarray analysis, allowing identification of differentially methylated DNA regions between different research samples, for example normal and tumor samples.

Example procedures for dsDNA digestion, immunoprecipitation, and amplification (WGA) are described in the MeDIP protocol, available from Roche Microarray Technical Support. Refer to page 5 for contact information.

Formats

The available formats for these two applications include:

- 385K (385,000 features)
- 4x72K (4 x 72,000 features)
- 2.1M (2.1 million features)
- 3x720K (3 x 720,000 features)

Array Workflow

The ChIP-chip and DNA Methylation protocols involve:

1. Performing quality control of experimental (IP) and control (input) samples to verify quality.
2. Independently labeling the samples using a NimbleGen Dual-Color DNA Labeling Kit.
3. Co-hybridizing the DNA to a NimbleGen ChIP-chip or DNA Methylation array using a NimbleGen Hybridization System.
4. Washing and drying the arrays, and then scanning them using a NimbleGen MS 200 Microarray Scanner.
5. Extracting array data using our DEVA software and analyzing the data using DEVA or SignalMap software.

Figure 1 lists the steps in the workflow for ChIP-chip and DNA Methylation arrays.

The corresponding estimated time for each step is based on processing one slide. When applicable, incubation times are indicated between process times in Figure 1.

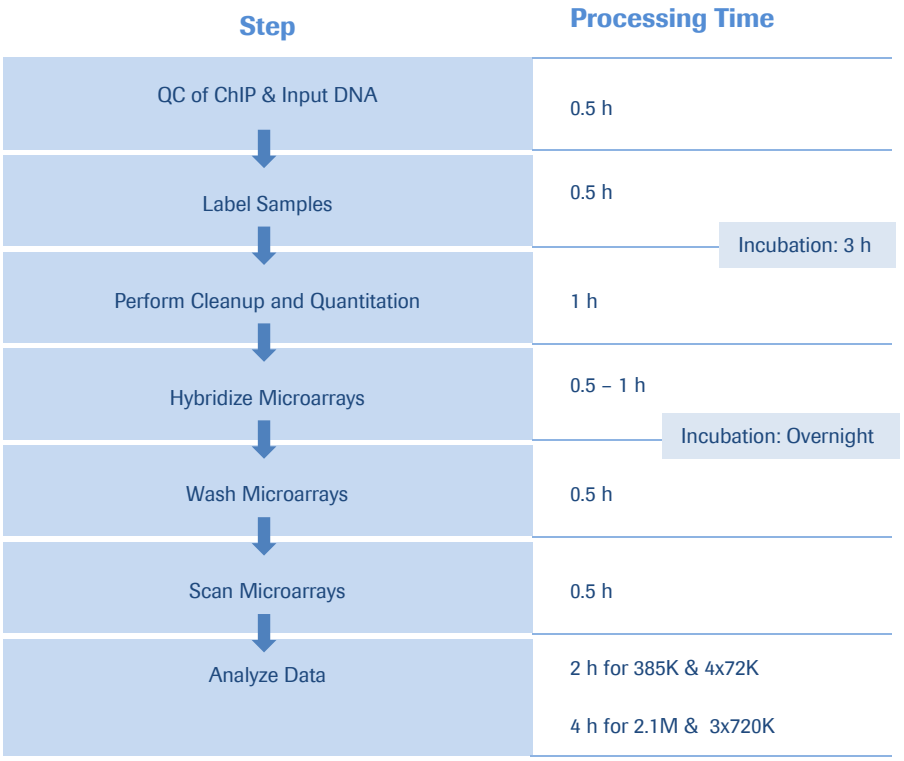


Figure 1: Workflow for NimbleGen Epigenetics Arrays.

What's New?

Version 1.0 of this *User's Guide* includes the following updates or modifications:

- Consolidation of ChIP-chip and DNA Methylation User's Guides into one document
- Instructions for data analysis in DEVA software
- Updated information for environmental requirements
- Updated information on array drying and scanning

This version should be used as a replacement for the following *User's Guides*:

NimbleGen Arrays User's Guide: ChIP-chip Arrays v6.2

NimbleGen Arrays User's Guide: DNA Methylation Arrays v7.2

To verify you are using the most up-to-date version of this *User's Guide* to process your arrays, go to *www.nimblegen.com/lit/*.

Components Supplied

Component	Description
NimbleGen Arrays	As ordered
NimbleGen Mixers	■ One X1 mixer *is ordered separately* from the 385K array ■ One X4 mixer is provided per 4x72K array ■ One HX1 mixer is provided per 2.1M array ■ One HX3 mixer is provided per 3x720K array
Mixer Port Seals or Mixer Multi-port Seals	For sealing fill and vent ports of NimbleGen mixers: ■ Mixer ports seals are provided with X1, HX1, and HX3 mixers ■ Mixer multi-port seals are provided with X4 mixers
CD/DVD	This *User's Guide* and NimbleGen design files are included in the Design Information CD/DVD.

Microarray Storage

NimbleGen arrays are packaged with desiccant and can be stored at +15 to +25°C for use by the expiration date. Once the seal is broken, store NimbleGen arrays in a desiccator at +15 to +25°C until use.

Environmental Requirements

Ozone

Cyanine dyes (Cy) are ozone sensitive. It is important to regularly monitor ozone levels in the lab environment and take the necessary precautions to maintain atmospheric ozone levels below 5 ppb (parts per billion).

The effect of ozone, at greater than 5 ppb, on Cy dye signal degradation can be reflected by a higher PMT gain, higher noise and lower signal to noise ratio in microarray data analysis.

To mitigate ozone problems in the lab environment Roche NimbleGen recommends using an ozone scrubber and ozone protection box during the wash and dry steps of the workflow, as well as over the microarray scanner.

Humidity

Cy dyes are humidity sensitive. Take the necessary precautions to keep humidity levels below 40%

Organic Solvents

Cy dyes are sensitive to organic solvents used in tissue fixation, such as formaldehyde and acetic acid. They are also sensitive to bleach and other cleaning chemicals. Maintain the necessary precautions to keep the Cy aways from these type of chemicals, and perform the array workflow in an organic solvents free environment.

Light

Cy dyes are light sensitive. Be sure to minimize light exposure of the dyes during use and store in the dark when not in use.

Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- Roche NimbleGen has found that using VWR water and DTT for all post-hybridization washes results in higher signal from Cy dyes.

  Reconstitute the DTT provided in the NimbleGen Wash Buffer Kit in a fume hood. *Chapter 4. Hybridization and Washing* provides details on how to reconstitute the DTT.
- Roche NimbleGen recommends using a NanoDrop Spectrophotometer for quantifying and characterizing nucleic acid samples because this instrument requires only 1.5 µl of sample for analysis.
- Perform all centrifugations at +15 to +25°C unless indicated otherwise.

Required Equipment, Labware & Consumables

You assume full responsibility when using the equipment, labware, and consumables described below. These protocols are designed for the specified equipment, labware, and consumables.

NimbleGen Equipment

Equipment	Supplier	Process Quantity	Catalog No.
NimbleGen Hybridization System 4*	Roche NimbleGen	4 slides	05 223 652 001 (110V)
			05 223 679 001 (220V)
NimbleGen MS 200 Microarray Scanner	Roche NimbleGen	48 slides	05 394 341 001

*NimbleGen Hybridization Systems include an accessory kit that contains a Precision Mixer Alignment Tool (PMAT), Mixer Disassembly Tool, Mixer Brayer, System Verification Assemblies, replacement O-rings, and forceps.

Software

Program	Supplier	Catalog No.
DEVA v1.0.2 or later	Roche NimbleGen	Available for free download from: *http://www.nimblegen.com/products/software/deva/index.html*
SignalMap v1.9	Roche NimbleGen	05 225 051 001 (Individual License)

Standard Laboratory Equipment

Equipment	Supplier	Catalog No.
Compressed Gas Nozzle	TeqCom	TA-N2-2000
DNA Vacuum Concentrator	Thermo Savant	
Desiccator	*Multiple Vendors*	
Electrophoresis System	*Multiple Vendors*	
Heat Block (capable of temperatures to +98°C)	*Multiple Vendors*	
Microcentrifuge (12,000 x g capability)	*Multiple Vendors*	
Microman M10 Pipette (recommended for 4x72K arrays)	Gilson	F148501
Microman M100 Pipette (recommended for 385K, 2.1M, and 3x720K arrays)	Gilson	F148504
Spectrophotometer	NanoDrop	ND-1000 or newer
Microarray Dryer which meets the following specifications: - Minimum of 4,000 revolutions per minute (rpm) - Capable of drying arrays in 30 seconds or less - Instrument is supplied with slide holder or cassette for 1x3 inch slide from the manufacturer	e.g. *VWR, ArrayIt®*	**VWR:** 93000-204 120V 93000-206 230V 93000-208 Slide Accessory **ArrayIT ®** MHC 110V MHC 220V
Thermocycler	*Multiple Vendors*	
Vortex Mixer	*Multiple Vendors*	

Consumables & Accessories Available from Roche NimbleGen

Component	Package Size / Process Quantity	Catalog No.
NimbleGen X1 Mixer (for 385K arrays; includes mixer port seals)	10 mixers	05 223 725 001
NimbleGen X4 Mixer (for 4x72K arrays; includes mixer multi-port seals)	10 mixers	05 223 733 001
NimbleGen HX1 Mixer (for 2.1M arrays; includes mixer port seals)	10 mixers	05 223 741 001
NimbleGen HX3 Mixer (for 3x720K arrays; includes mixer port seals)	10 mixers	05 223 750 001
NimbleGen Dual-Color DNA Labeling Kit Contents: ■ PCR Grade Water (2 x vial 1) ■ Random Primer Buffer (2 x vial 2) ■ Cy3 Random Nonamers (vial 3) ■ Cy5 Random Nonamers (vial 4) ■ Klenow Fragment (3'->5' exo-) 50 U/µl (2 x vial 5) ■ dNTP Mix (10 mM each dNTP) (2 x vial 6) ■ Stop Solution (2 x vial 7)	48 reactions (24 Cy3 and 24 Cy5 labeling reactions)	06 370 250 001
NimbleGen Dual-Color DNA Labeling Kit Contents: ■ PCR Grade Water (2 x vial 1) ■ Random Primer Buffer (2 x vial 2) ■ Cy3 Random Nonamers (4 x vial 3) ■ Cy5 Random Nonamers (4 x vial 4) ■ Klenow Fragment (3'->5' exo-) 50 U/µl (2 x vial 5) ■ dNTP Mix (10 mM each dNTP) (2 x vial 6) ■ Stop Solution (2 x vial 7)	192 reactions (96 Cy3 and 96 Cy5 labeling reactions)	06 370 381 001

Component	Package Size / Process Quantity	Catalog No.
NimbleGen Hybridization Kit Contents: ■ 2X Hybridization Buffer (vial 1) ■ Hybridization Component A (vial 2) ■ Alignment Oligo[1] (vial 3)	■ 100 hybridizations using 385K arrays ■ 160 hybridizations using 4x72K arrays ■ 40 hybridizations using 2.1M arrays ■ 102 hybridizations using 3x720K arrays	05 583 683 001
NimbleGen Hybridization Kit, LS (Large Scale) Contents: ■ 2X Hybridization Buffer (3 x vial 1) ■ Hybridization Component A (3 x vial 2) ■ Alignment Oligo[1] (3 x vial 3)	■ 305 hybridizations using 385K arrays ■ 488 hybridizations using 4x72K arrays ■ 122 hybridizations using 2.1M arrays ■ 306 hybridizations using 3x720K arrays	05 583 934 001
NimbleGen Sample Tracking Control Kit Contents: Sample Tracking Controls[2]	■ 480 hybridizations using 4x72K arrays ■ 300 hybridizations using 3x720K arrays	05 223 512 001
NimbleGen Wash Buffer Kit Contents: ■ 10X Wash Buffer I (2 x vial 1) ■ 10X Wash Buffer II (vial 2) ■ 10X Wash Buffer III (vial 3) ■ DTT (2 x vial 4) ■ Nuclease-free Water (3 x vial 5)	20 washes (processing up to 12 slides per wash)	05 584 507 001
NimbleGen Array Processing Accessories Contents: ■ Slide Rack ■ Wash Tanks ■ Slide Containers		05 223 539 001

1 The Alignment Oligo is a mixture of Cy3 and Cy5 labeled 48 mer oligonucleotides that hybridize to alignment features on NimbleGen arrays. It is required for proper extraction of array data from the scanned image.

2 Twelve Sample Tracking Controls (STCs) are provided. Each STC is a Cy3-labeled 48 mer oligonucleotide. When a unique STC is added to each sample before hybridization to a multiplex array, the STC can be used to confirm that the correct sample was hybridized to each array.

Reagents/Consumables Purchased from Other Vendors

Component	Supplier	Package Size	Catalog No.
β-Mercaptoethanol	Sigma Aldrich	25 ml	M3148
Absolute Ethanol	Sigma Aldrich	500 ml	E702-3
Compressed Nitrogen or Argon Gas (for cleaning array surface)*	*Multiple Vendors*		
CP10 Pipette Tips (for 4x72K arrays)	Gilson	192 tips	F148412
		960 tips	F148312
CP100 Pipette Tips (for 385K, 2.1M, and 3x720K arrays)	Gilson	192 tips	F148414
		960 tips	F148314
Isopropanol	Sigma Aldrich	500 ml	I-9516
Water: reagent grade, ACS, nonsterile, type 1	VWR	2.5 gallon	RC915025
Cotton Swabs	*Multiple Vendors*		

* Roche NimbleGen recommends using a compressed gas nozzle to gently blow compressed nitrogen or argon gas across arrays to remove any dust or debris. The use of canned compressed air for this purpose is not recommended and could compromise array and data quality.

Chapter 2. Preparing Samples & Performing QC

Chapter 2 describes sample requirements and the sample QC protocol for NimbleGen Epigenetic experiments.

Preparing Samples

ChIP-chip

High-quality experimental (IP) and control (input) DNA are required to obtain optimally abeled samples for array hybridization. The NimbleGen sample preparation protocol for ChIP-chip is available upon request from Roche Microarray Technical Support.

The success of the IP reaction depends on using antibodies validated with immunoprecipitation. Not all antibodies work well for this method.

DNA Methylation

High-quality DNA generated using the MeDIP (methylated DNA immunoprecipitation) method is required to obtain optimally labeled samples for array hybridization. The NimbleGen sample preparation protocol for DNA methylation is available upon request from Roche Microarray Technical Support.

The success of the IP reaction depends on the amount of 5-methyl cytidine antibody used. Amounts should be determined empirically by titration.

The sample requirements are listed below:

- Roche NimbleGen recommends starting with the following sample amounts for each hybridization. If your experimental (IP) sample quantity is less than the amount listed, amplify the experimental (IP) and control (input) samples using the Sigma GenomePlex Complete WGA 2 Kit (Catalog No. WGA2-50RXN) before labeling. Then purify samples using the Qiagen QIAquick PCR Purification Kit (Catalog No. 28106).

Starting Sample Amount Recommendations	385K Array	Each Sample for a 4x72K Array	2.1M Array	Each Sample for a 3x720K Array
Experimental (IP) Sample	1.5 µg	1.5 µg	1.5 µg	1.5 µg
Control (Input) Sample	1.5 µg	1.5 µg	1.5 µg	1.5 µg

For optimal results, samples should meet the following criteria:

- A significant majority of the DNA ≥ 200 nucleotides in size.
- A concentration of approximately 250 ng/µl to 1,000 ng/µl in PCR grade water or 1X TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 7.5 - 8.0).
- An A260/A280 ≥ 1.7 and A260/A230 ≥ 1.6.

Performing Sample QC

1. Transfer 200 ng of each sample to a sterile microcentrifuge tube. Store the remainder of your sample set at -15 to -25°C until required for labeling.
2. Analyze the samples using the Agilent Bioanalyzer and RNA 6000 Nano Assay Reagent Kit or by agarose gel electrophoresis.
3. Review Bioanalyzer traces (Figure 2 and Figure 3) or agarose gels (Figure 4) for sample degradation. Degraded samples detected using the Bioanalyzer appear as significantly lower intensity traces with the main peak area shifted to the left with typically more noise in the trace.

Samples exhibiting degradation should not undergo labeling and hybridization due to the risk of poor results.

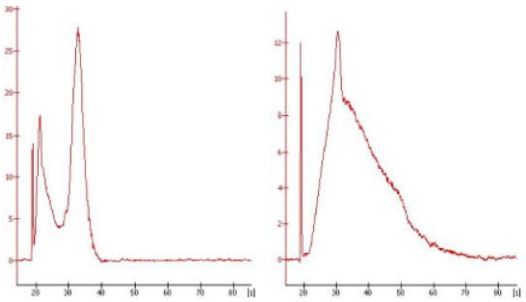


Figure 2: Examples of Bioanalyzer Traces of Nondegraded Samples

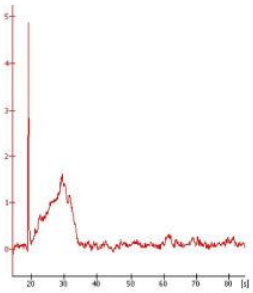


Figure 3: Example of Bioanalyzer Trace of a Degraded Sample

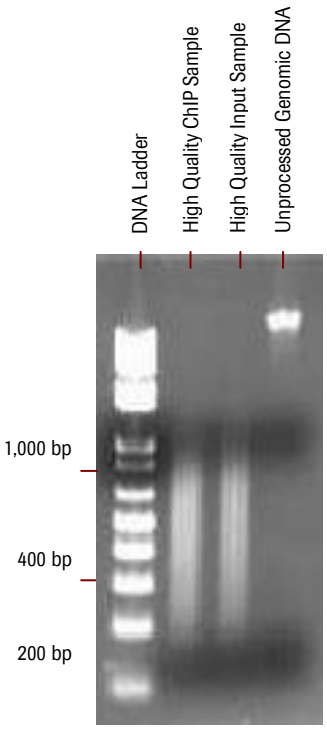


Figure 4: Example of Agarose Gel Electrophoresis for Samples of Good Quality

Notes

Chapter 3. Labeling Samples

Chapter 3 describes how to label your experimental (IP) and control (input) samples using the NimbleGen Dual-Color DNA Labeling Kits.

Labeling Guidelines

Be aware of the following when using the NimbleGen Dual-Color Labeling Kits:

- Aliquot dNTPs and Cy primers into single-use amounts.
- Stop Solution could precipitate. Vortex or heat if necessary.

Most researchers performing MeDIP include a control (input) sample. However, some researchers will perform another two-color array experiment, running the control (input) sample against a non-specific binding sample to test for false positives.

- A small aliquot of the starting DNA is carried through the labeling protocol and used as a reference sample.
- Non-Specific Binding (NSB) samples are IP reactions carried out where the primary antibody (anti-5-methyl-cytosine here) is omitted or substituted with pre-immune or non-specific immunoglobulin G. NSB samples are used to test the level of enrichment specific to the IP antibody.

Potential arrays run in parallel if using NSB samples are as follows:

Test array = Cy5-labeled experimental (IP) sample/Cy3-labeled control (input) sample

False positive array = Cy5-labeled NSB sample/Cy3-labeled control (input) sample

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

Performing Sample Labeling

To label samples, follow these steps:

1. Prepare the following solution in a 15 ml tube:

Random Primer Buffer	All Array Formats	Notes
Random Primer Buffer (vial 2)	2,200 µl	Prepare fresh buffer each time primers are resuspended.
β-Mercaptoethanol*	4 µl	
Total	2,204 µl	

* Do not use bottles of β-Mercaptoethanol that have been opened for more than 6 months.

2. Briefly centrifuge Cy3 Random and Cy5 Random Nonamers (vials 3 and 4, respectively) because some of the product could have dislodged during shipping. Dilute the primers in 1,050 µl each of Random Primer Buffer with β-Mercaptoethanol. Aliquot to 40 µl individual reaction volumes in 0.2 ml thin-walled PCR tubes and store at -15 to -25°C, protected from light.

Do not store diluted primers longer than 4 months.

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

Component	All Array Formats	
	Experimental (IP) Sample	Control (Input) Sample
Sample prepared in Chapter 2	1 µg	1 µg
Diluted Cy3 Random Nonamers from step 2		40 µl
Diluted Cy5 Random Nonamers from step 2	40 µl	
PCR Grade Water (vial 1)	To volume (80 µl)	To volume (80 µl)
Total	**80 µl**	**80 µl**

* 2.1M arrays require 34 µg labeled DNA per sample for each array, and in general, enough material is produced when setting up the labeling reactions as described above. However, if insufficient labeled material is produced, repeat each labeling reaction and pool the appropriate labeled samples before hybridization.

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

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

5. Prepare the following dNTP/Klenow master mix for each sample prepared in step 4.

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
dNTP Mix (10 mM each dNTP) (vial 6)	10 µl
PCR Grade Water (vial 1)	8 µl
Klenow Fragment (3'->5' exo-) 50 U/µl (vial 5)	2 µl
Total	**20 µl**

6. Add 20 µl of the dNTP/Klenow master mix prepared in step 5 to each of the denatured samples prepared in step 4. Keep on ice.

Component	All Array Formats	
	Experimental (IP) Sample	Control (Input) Sample
Reaction volume from step 4	80 µl	80 µl
dNTP/Klenow Master Mix from step 5	20 µl	20 µl
Total	**100 µl**	**100 µl**

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

   
   Do not vortex after addition of Klenow.

8. Quick-spin to collect contents in bottom of the tube.
9. Incubate for 3 hours at +37°C in a thermocycler with heated lid, protected from light.
10. Stop the reaction by addition of the Stop Solution.

Component	All Array Formats	
	Experimental (IP) Sample	Control (Input) Sample
Reaction volume from step 6	100 µl	100 µl
Stop Solution (vial 7)	21.5 µl	21.5 µl
Total	**121.5 µl**	**121.5 µl**

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

Component	All Array Formats	
	Experimental (IP) Sample	Control (Input) Sample
Reaction volume from step 10	121.5 µl	121.5 µl
Isopropanol	110 µl	110 µl
Total	**231.5 µl**	**231.5 µl**


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

12. Vortex well. Incubate for 10 minutes at +15 to +25°C, protected from light.
13. 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.
14. Rinse pellet with 500 µl 80% ice-cold ethanol. Dislodge pellet from tube wall by pipetting a few times.
15. Centrifuge at 12,000 x g for 2 minutes. Remove supernatant with a pipette.
16. Dry contents in a DNA vacuum concentrator on low heat until dry (approximately 5 minutes), protected from light.
17. STOP POINT: Proceed to step 18, or store labeled samples at -15 to -25°C (up to 1 month), protected from light.
18. Spin tubes briefly prior to opening. Rehydrate each pellet in 25 µl PCR Grade Water (vial 1) per reaction.
19. Vortex for 30 seconds and quick-spin to collect contents in bottom of the tube. Continue to vortex or let sit at +15 to +25°C, protected from light, for approximately 5 minutes or until the pellet is completely rehydrated, then vortex again and quick-spin.

20. Quantitate each sample using the following formula:

$$\text{Concentration } (\mu g/ml) = A_{260} \times 50 \times \text{Dilution Factor}$$

If using a NanoDrop Spectrophotometer, refer to the manufacturer's instructions to ensure accurate quantitation.

21. Based on the concentration, calculate the volume of the 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 1.5 ml tube:

Sample Requirements	385K Array	4x72K Array	2.1M Array	3x720K Array
Experimental (IP) Sample	6 µg	4 µg	34 µg*	15 µg
Control (Input) Sample	6 µg	4 µg	34 µg*	15 µg

* If the 34 µg was not obtained from the 1 labeling reaction, perform a second labeling reaction and combine the labeled products. Alternatively, the hybridization can be performed with as little as 24 µg.

22. Dry contents in a DNA vacuum concentrator on low heat, protected from light.

23. STOP POINT: Proceed to *Chapter 4*, or store labeled samples at -15 to -25°C (up to 1 month), protected from light.

Chapter 4. Hybridizing & Washing

Chapter 4 describes the NimbleGen protocol for sample hybridization and washing.

Before You Begin

Requirements

- For the hybridization protocol, you must use a NimbleGen Hybridization System. Refer to the *NimbleGen Hybridization System User's Guide* for specific instructions on its use.
- For the hybridization protocol, you must adhere a NimbleGen mixer to the microarray slide. Refer to the package label to identify the mixer design. Some instructions in the protocol are specific to the mixer design.
- The Alignment Oligo and Sample Tracking Controls (STCs) provided in the NimbleGen Hybridization and Sample Tracking Control Kits, respectively, are labeled with Cy dyes, which are sensitive to photobleaching and freeze-thawing. After thawing stock tubes for the first time, aliquot the Alignment Oligo and STCs into single-use volumes and freeze at -15 to -25°C. Protect tubes from light.

Step 1. Preparing Samples for Hybridization

1. Turn on the Hybridization System and set it to +42°C. With the cover closed, allow at least 3 hours for the temperature to stabilize.

   Be aware that the temperature of the Hybridization System could fluctuate during stabilization.

2. Resuspend the dried sample pellet in Sample Tracking Control according to the following table. Use a unique STC to resuspend each sample to be hybridized. Record which STC is used for each sample.

   If you are not using Sample Tracking Controls, resuspend the dried sample pellet in the equivalent volume of water.

Component	385K Array	Each Sample for a 4x72K Array	2.1M Array	Each Sample for a 3x720K Array
Reagent for resuspension	VWR Water	Sample Tracking Control	VWR Water	Sample Tracking Control
Volume to add to Cy-labeled Sample from step 23 in Chapter 3	5 µl	3.3 µl	12.3 µl	5.6 µl

3. Vortex well and spin to collect contents in bottom of the tube.
4. Vortex each component from a NimbleGen Hybridization Kit (approximately 15 seconds) and spin to collect contents in the bottom of the tube

5. Prepare the hybridization solution master mix according to the following table. For 4x72K and 3x720K arrays, the amount listed is sufficient to hybridize all arrays on one slide. To hybridize multiple slides, adjust the amounts accordingly.

Hybridization Solution Master Mix to Hybridize a Single Slide	385K Array	4x72K Array	2.1M Array	3x720K Array
2X Hybridization Buffer (vial 1)	11.8 µl	29.5 µl	29.5 µl	35 µl
Hybridization Component A (vial 2)	4.7 µl	11.8 µl	11.8 µl	14 µl
Alignment Oligo (vial 3)	0.5 µl	1.2 µl	1.2 µl	1.4 µl
Total	**17 µl**	**42.5 µl**	**42.5 µl**	**50.4 µl**

6. Add the appropriate amount of hybridization solution to each sample pair according to the following table:

Component	385K Array	Each Sample Pair for a 4x72K Array	2.1M Array	Each Sample Pair for a 3x720K Array
Resuspended sample from step 2	5 µl	3.3 µl	12.3 µl	5.6 µl
Hybridization solution from step 4	13 µl	8.7 µl	31.7 µl	14.4 µl
Total	**18 µl**	**12 µl**	**44 µl**	**20 µl**

7. Vortex well (approximately 15 seconds) and spin to collect contents in bottom of the tube. Incubate at +95°C for 5 minutes, protected from light.

8. Place tubes at +42°C (in the Hybridization System sample block or heat block) for at least 5 minutes or until ready for sample loading. Vortex and spin prior to loading.

Step 2. Preparing Mixers

1. Locate the appropriate mixer. Remove from its package.

For best results, use a compressed gas nozzle to gently blow compressed nitrogen or argon gas across the mixer and slide to remove any dust or debris. The use of canned compressed air for this purpose is not recommended and could compromise array and data quality.

Load samples within 30 minutes of opening the vacuum-packaged mixer to prevent the formation of bubbles during loading and/or hybridization.

Array Format	Mixer
385K array	X1 mixer
4x72K array	X4 mixer
2.1M array	HX1 mixer
3x720K array	HX3 mixer

2. Position the Precision Mixer Alignment Tool (PMAT) with its hinge on the left. Open the PMAT (Figure 5).
3. Snap the mixer onto the two alignment pins on the lid of the PMAT with the tab end of the mixer toward the inside hinge and the mixer's adhesive gasket facing outward (Figure 5).
4. While pushing back the plastic spring with a thumb, place the slide in the base of the PMAT so that the barcode is on the right and the corner of the slide sits against the plastic spring. The NimbleGen logo and barcode number should be readable.

   Remove your thumb and make sure the spring is engaging the corner of the slide and the entire slide is registered to the edge of the PMAT to the rightmost and closest to you. In addition, be sure that the slide is lying flat against the PMAT.

   While holding the slide by the edges, place the slide into the PMAT base using gloved fingers. The use of metal forceps may damage the glass slide and create a potentially hazardous condition (for example, broken glass) for the operator.

   Take care to align the slide correctly in the PMAT. Incorrectly aligned slides may result in inaccurate attachment of the mixer and may affect the array features, or may not fit well into the Mixer Disassembly Tool used to remove the mixers after hybridization.
5. Gently blow compressed nitrogen or argon gas across the mixer and slide to remove dust.
6. Using forceps, remove the backing from the adhesive gasket of the mixer and close the lid of the PMAT so that the gasket makes contact with the slide.
7. Lift the lid by grasping the long edges of the PMAT while simultaneously applying pressure with a finger through the window in the lid of the PMAT to free the mixer-slide assembly from the alignment pins.

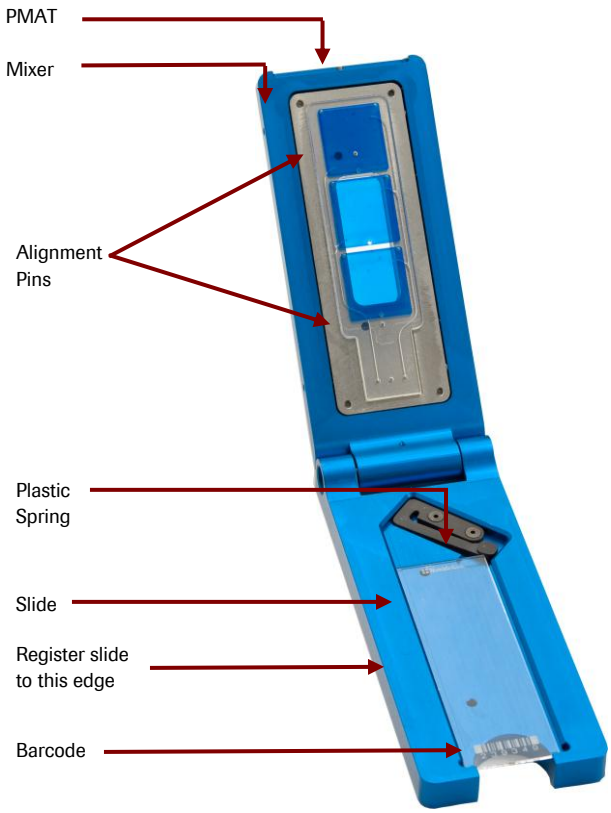


Figure 5: PMAT with HX3 Mixer and Slide. For photographic purposes only, blue coloring was used to show the location of the mixer's hybridization chambers. The hybridization chambers of the mixer you receive will not be blue.

8. Remove the mixer-slide assembly from the PMAT.
9. Place the mixer-slide assembly on the back of a +42°C heating block for 5 minutes to facilitate complete adhesion of the mixer to the slide.
10. Rub the Mixer Brayer over the mixer with moderate pressure to adhere the adhesive gasket and remove any bubbles.
    - For X1 and HX1 mixers, start in the center of the array and rub outward.
    - For X4 and HX3 mixers, first use a corner of the Mixer Brayer to rub the borders between the arrays and then rub around the outside of the arrays.

    The adhesive gasket will become clear when fully adhered to both surfaces.
11. Place the mixer-slide assembly in the slide bay of the Hybridization System.

Step 3. Loading & Hybridizing Samples

Refer to the appropriate diagram below when loading samples and applying port seals.

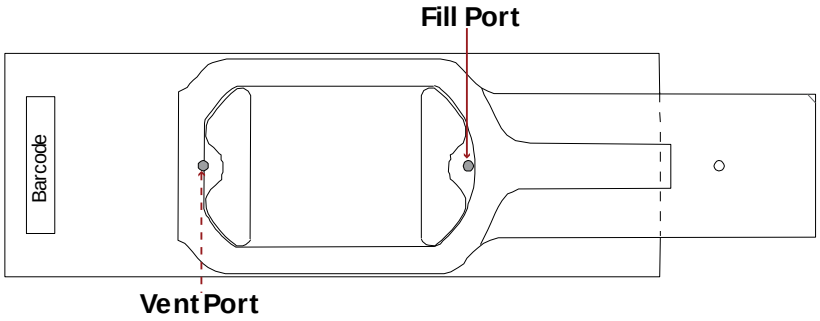


Figure 6: **X1 Mixer and Slide for a 385K Array**

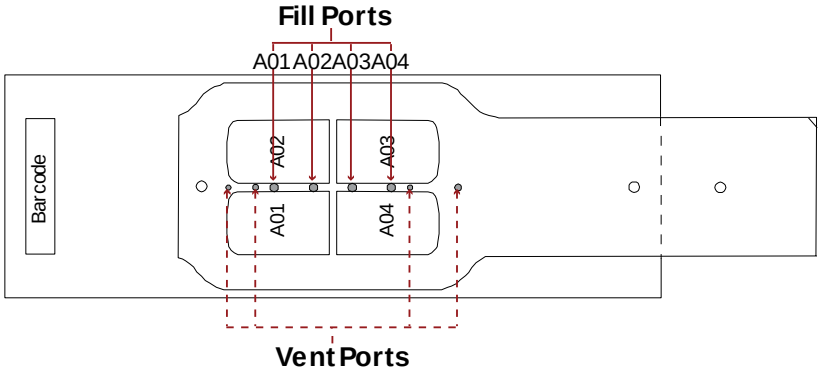


Figure 7: **X4 Mixer and Slide for a 4x72K Array**

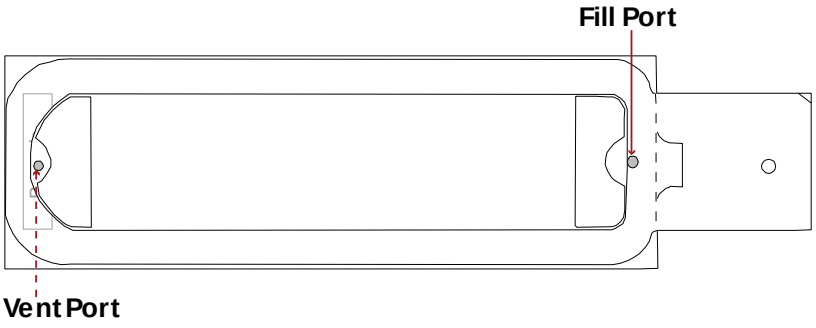


Figure 8: **HX1 Mixer and Slide for a 2.1M Array**

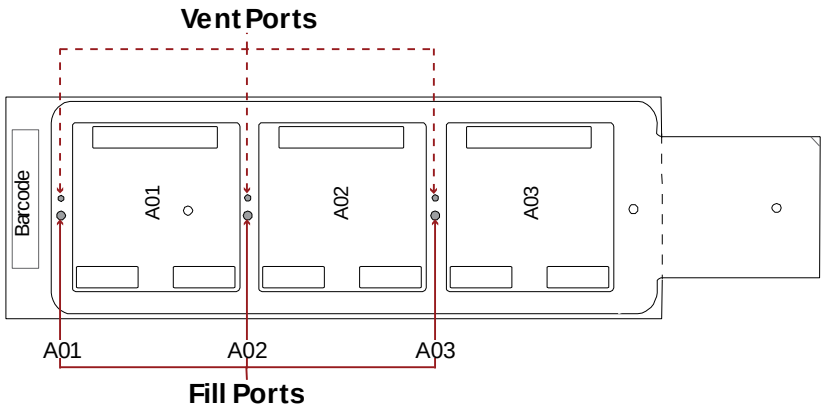


Figure 9: HX3 Mixer and Slide for a 3x720K Array

1. Keep the following in mind before loading sample:

   - When pipetting the sample before loading, leave residual volume in the sample tube to avoid introducing bubbles. The volumes listed in the table below allow for a residual volume.
   - After aspirating the designated sample volume, inspect the pipette tip for air bubbles. Dispense and reload the pipette if bubbles exist.

   Keep the following in mind when loading sample:

   - Keep the pipette tip perpendicular to the slide to avoid possible leakage at the fill port.
   - Apply gentle pressure of the tip into the port to ensure a tight seal while loading the sample.

Component	385K Array	4x72K Array	2.1M Array	3x720K Array
Sample Loading Volume	16 µl	8 µl	41 µl	18 µl
Pipette Tip	CP100	CP10*	CP100	CP100

* The CP10 tip is thin and flexible. Place the thumb and forefinger of your free hand on the tip to guide it into the port.

2. Using the appropriate Gilson Microman pipette, dispense the appropriate sample volume into the fill port. Load samples and seal mixer ports as described below for each array format:

For 385K, 2.1M, and 3x720K arrays:

a. Load sample into a fill port. Dry any overflow from the fill and vent ports with a cotton swab after loading the array.

   For 3x720K arrays, it is not unusual for small bubbles to form in the corners of the mixer-slide assembly during loading. These bubbles will dissipate upon mixing and will not compromise the data.

b. On X1 or HX1 mixers, use one mixer port seal to cover the fill port and another to cover the vent port. On HX3 mixers, use one mixer port seal to cover both the fill and vent ports, filling and sealing one chamber at a time. To adhere, press the mixer port seal, using uniform pressure across the seal to adhere.

c. Use forceps to press the mixer port seal around the fill and vent ports to ensure it is adhered in those areas.

For 4x72K arrays:

a. Load sample into the A01 fill port. Dry any overflow from the fill and vent ports with a cotton swab after loading. Repeat loading samples into the A02 - A04 fill ports, using a fresh cotton swab for drying the ports for each array.

b. On X$ mixers, use one mixer multi-port seal to cover all fill and vent ports. To adhere, press the mixer multi-port seal, using uniform pressure across the seal to adhere.

c. Use forceps to press the mixer multi-port seal around the fill and vent ports to ensure it is adhered in those areas.

3. Close the bay clamp.
4. Turn on the Mixing Panel on the Hybridization System, set the mix mode to B, and press the mix button to start mixing. Confirm that the Hybridization System recognizes the slide in each occupied bay (its indicator light becomes green).
5. Approximately 10 minutes after starting the Hybridization System:
   - Ensure the mix mode is set to B.
   - Ensure a green light is displayed for all occupied stations.
6. Hybridize sample at +42°C to the array(s) for 16 - 20 hours.

Step 4. Washing Hybridized Arrays

To ensure high quality data, it is important to proceed through all the washing and drying steps without interruption. If using a microarray dryer that dries one slide at a time, wash only one slide at a time.

1. Locate the components of the NimbleGen Wash Buffer Kit and NimbleGen Array Processing Accessories (refer to page 12).

Prior to the first use of the Wash Buffer Kit, reconstitute the DTT. In a fume hood, prepare 1 M DTT solutions by adding 1.2 ml of water (vial 5) to each tube of dry DTT (vial 4). After reconstitution, store the 1 M DTT solutions at -15 to -25°C.

2. Before removing the mixer-slide assemblies from the Hybridization System, prepare Washes I, II, and III according to the following tables. Note that you prepare two containers of Wash I.

Washing Multiple Slides	Wash I (user-supplied dish[1])	Washes I, II, and III (wash tank[2])
VWR Water	243 ml	243 ml
10X Wash Buffer I, II, or III (vial 1, 2, or 3)	27 ml	27 ml
1 M DTT solution from step 1	27 µl	27 µl
Total	**270 ml**	**270 ml**

Washing One Slide	Wash I (user-supplied dish[1])	Washes I, II, and III (slide container[2])
VWR Water	243 ml	24.3 ml
10X Wash Buffer I, II, or III (vial 1, 2, or 3)	27 ml	2.7 ml
1 M DTT solution from step 1	27 µl	2.7 µl
Total	**270 ml**	**27 ml**

1 Ensure that this dish is shallow and wide enough to accommodate the mixer-slide assembly loaded in the Mixer Disassembly Tool. This dish must also be small enough to ensure that the Mixer Disassembly Tool is completely submerged in the wash solution.

2 If washing multiple slides, prepare the washes in the wash tanks. If washing only one slide, prepare the washes in the slide containers.

3. To facilitate the removal of the mixer, heat the shallow dish containing Wash I to +42°C +/-2°C. Roche NimbleGen recommends measuring the temperature of Wash I at every use. Keep the remaining three wash solutions at room temperature (+15 to +25°C).

4. Insert the Mixer Disassembly Tool into the shallow dish containing 42°C +/-2°C Wash I. If you will be washing multiple slides, insert a slide rack into the wash tank containing Wash I at +15 to +25°C.

5. Remove a mixer-slide assembly from the Hybridization System and load it into the Mixer Disassembly Tool immersed in the shallow dish containing 42°C+/-2°C Wash I.

Do not allow the mixer-slide assembly to cool before removing the mixer. Keep power on to the Hybridization System's heat block and mixer system during mixer-slide disassembly, and transfer each mixer-slide assembly one at a time to 42°C +/-2°C Wash I for immediate removal of the mixer.

6. With the mixer-slide assembly submerged, carefully peel the mixer off the slide. It is important to hold the Mixer Disassembly Tool flat while removing the mixer and to avoid any horizontal movement or scraping with the mixer across the slide. Do not touch the array surface of the slide.

The mixer is extremely flexible. Peel the mixer off slowly to avoid breaking the slide.

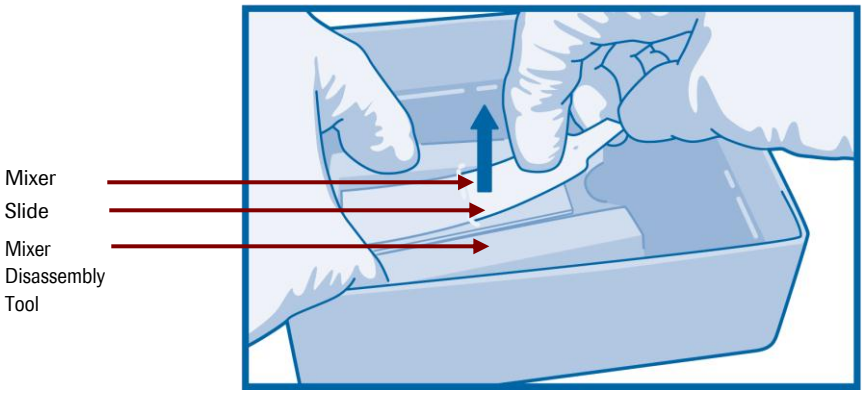


Figure 10: Using the Mixer Disassembly Tool to Remove a Slide from a Mixer

7. Working quickly, discard the mixer and remove the slide from the Mixer Disassembly Tool.

8. Gently agitate the slide for 10 - 15 seconds in the shallow dish containing 42°C +/-2°C Wash I to quickly remove the hybridization buffer.

   It is important for achieving good array uniformity to quickly and evenly wash the hybridization buffer off the slide surface as soon as the mixer is removed.

9. If washing multiple slides, transfer the slide with the barcode at the top into a slide rack (Figure 11) in the wash tank that contains room temperature Wash I. If washing one slide, transfer the slide into a slide container that contains room temperature Wash I. Agitate vigorously for 10 - 15 seconds.

   Slide rack users: To ensure high quality data, make sure the microarray area of the slide remains wet at all times during all wash steps.

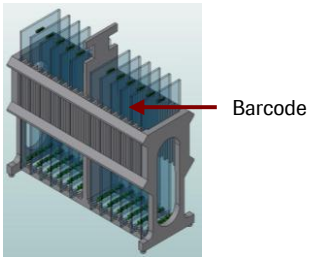


Figure 11: Insert Slides with the Barcode at the Top into the Slide Rack

   If you are using a microarray dryer that dries multiple slides at a time, repeat steps 4 to 9 (checking that the wash buffer temperature has not cooled below 40°C) until you have removed the mixer from all slides to wash. Load each slide into the slide rack with the array facing the same direction.

10. Wash for an additional 2 minutes in room temperature Wash I with vigorous, constant agitation. If washing multiple slides, move the rack up and down with enough agitation to make foam appear. If washing one slide, shake the slide container at least 20 times every 10 seconds.

    At several times during the wash, rock the wash tank so the wash solution covers and cleans the top of the slide(s).

11. Quickly blot the rack, or edges of the slide if only washing one slide, several times using paper towels to minimize buffer carryover.
12. Transfer the slide(s) to Wash II and wash for 1 minute with vigorous, constant agitation. If washing multiple slides, rock the wash tank so the wash solution covers and cleans the tops of the slide(s).

    Do not allow slides to dry between wash steps.
13. Transfer the slide(s) to Wash III and wash for 15 seconds with vigorous, constant agitation. If washing multiple slides using the slide rack, rock the wash tank so the wash solution covers and cleans the tops of the slide(s).
14. Remove the slide(s) from Wash III. Immediately spin dry in a NimbleGen Microarray Dryer or other microarray dryer per the manufacturer's recommendation for 30 seconds.

    If using a microarray dryer capable of holding more than one slide, ensure that the rotator is balanced prior to drying.

    To help mitigate ozone and humidity effects on Cy degradation it is important to shorten the dry time by using an array dryer that can dry the slides to completion in a 30 seconds. It is also important to remove the slides promptly after they have been dried to avoid exposure to the humidity that remains inside the instrument.
15. Remove the slide(s) from microarray dryer. Blot dry the edges with lint-free paper to remove any residual moisture from the edges of the slide.
16. Proceed immediately to the steps for scanning the array(s) in *Chapter 5*.

    If you cannot immediately scan your slides after washing, keep them in their original slide case in a dark desiccator until you are ready to scan them.

Chapter 5. Scanning Two-Color Arrays

Chapter 5 describes the protocol for scanning two-color NimbleGen arrays with the MS 200 Microarray Scanner and the MS 200 Data Collection Software.

Before You Begin

Before starting these procedures, review the information in the *NimbleGen MS 200 Microarray Scanner Operator's Manual* (available at *www.nimblegen.com/products/instruments/* under *Literature*) or the online help available via the MS 200 Data Collection Software. These materials provide more detailed instructions on using the scanner, control unit (computer), and software than provided in this chapter.

When handling slides, wear powder-free gloves and use care to touch only the slide's edges.

To mitigate ozone problems in the lab environment Roche NimbleGen recommends placing an ozone scrubber and ozone protection box over the scanner during scanning time. At ozone levels of less than 5 ppb, it is possible to rescan the arrays without any photobleaching effects. However, in the presence of greater than 5 ppb of ozone, Cy dye signal degradation can occur over time, or during rescanning of the same slides.

Step 1. Starting the Scanner

1. Start the control unit and log into your user account.

   Account: **msOperator**
   Password: **1-msOperator**

2. Turn on the scanner using the power switch on the left side. Ensure the main power switch on the left rear of the scanner is also in the on position.
3. Using a compressed gas nozzle, gently blow compressed nitrogen or argon gas across the slide to remove any dust or debris from the array. Do not use canned compressed air for this purpose as it could compromise data quality.
4. Insert slides into the Slide Magazine
5. Insert the slide magazine with loaded slides into the scanner

Step 2. Starting the Software & Turning on the Lasers

1. Launch the MS 200 Data Collection Software. Ensure the software has completely loaded before continuing.
2. Turn on the lasers by clicking the **Green Laser** and **Red Laser** buttons in the Laser Control (Figure 12).

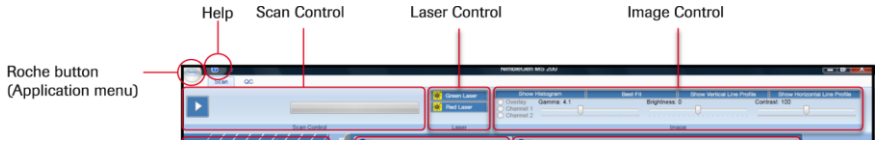


Figure 12: Top of Data Collection Workspace, showing Scan Control, Laser Control, and Image Control

3. Confirm the Magazine Control pane (Figure 13) displays a green box in the *Slide Present* field for each slide loaded into the slide magazine.
4. Confirm the *Select Scan* check box is selected for each slide to scan.

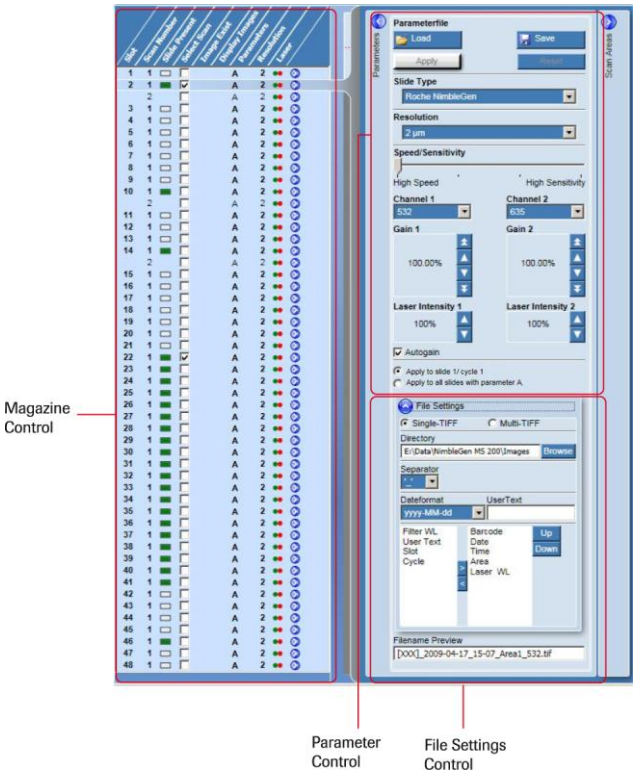


Figure 13: Magazine Control, Parameter Control, and File Settings Control

Step 3. Set Scan Parameters

Use the Parameter Control (Figure 13) to set the parameters to use for scanning.

1. Click the **Open Parameter Control** button ( ) to display the Parameter Control pane.
2. Use the following recommended default parameters unless adjustments are determined to be necessary:

Parameter	Default Setting
Slide type	Roche NimbleGen
Speed/Sensitivity	High Speed
Channel 1	532
Channel 2	635
Laser Intensity 1	100%
Laser Intensity 2	100%
Autogain	Selected
Apply to slide 1/cycle 1	Selected

3. Select the desired scanning resolution.
   - For 2.1M and 3x720K arrays, 2 µm resolution is recommended for best results.
   - For 385K and 4x72K arrays, 5 µm resolution is sufficient.

   Resolutions higher than 5 µm may be used for pre-scanning but are not recommended for generating data for analysis.
4. Click **Apply** to save any changes to the settings.
5. *Optional:* To save settings to a parameters file ensure the Parameter Control window is closed and then click **Save**.
6. *Optional:* To process the slide multiple times using the same or different scanning parameters, refer to the *NimbleGen MS 200 Microarray Scanner Operator's Manual*.

File Settings Control Pane

Use the File Settings Control (Figure 13) to specify image file settings:

1. Click **Open/Close File Settings** button ( ) to open the File Settings Control pane.
2. Choose the **Single-TIFF** option button to generate one image file in Tagged Image File Format (TIFF, .tif) per channel.
3. Confirm the path to which the images files will be saved. The default path is E:\Data\NimbleGen MS 200. To change the path, click **Browse** to specify a location in the directory and click **OK** to confirm.

4. Determine the file name by using the annotation list to add or change annotations to include in file names.

   If you will be using DEVA software for data analysis, specify and order the annotations as follows:

   <Barcode>_<Other Annotations>_<Laser WL>.tif

   "Barcode" must be the first annotation and "Laser WL" must be the last annotation. "WL" represents wavelength. To change the order of the annotations, select the annotation and click the **Up** or **Down** button. If you include user text, type the text in the *User Text* field

   For example, a file name assigned by the MS 200 Data Collection Software might be as follows:

   100000_Sample12345_532.tif

   where the annotations are "Barcode," "User Text," and "Laser WL."

5. Click **Apply** to confirm settings.

6. *Optional:* To save the file settings to a file, click **Save** in the Parameters Control (Figure 13).

Area Definition Control Pane

Use the Area Definition Control (Figure 14; denoted as *Scan Areas* in the software interface) to set scan, barcode, and autogain areas.

1. Click **Open/Close Area Definition Control** button ( )Figure 14.

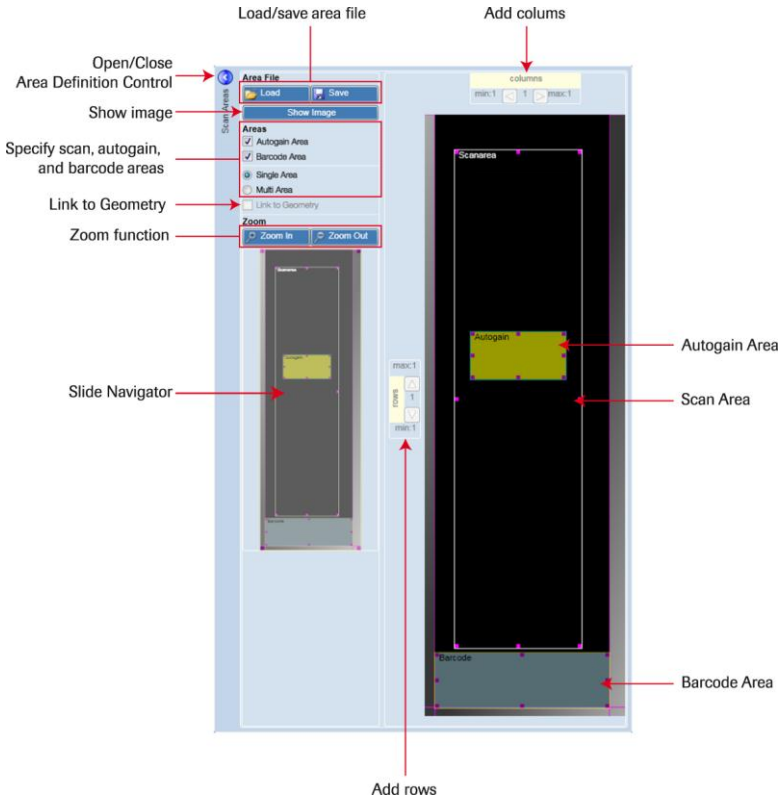


Figure 14: Components of the Area Definition Control

2. Check the **Barcode area** box.
3. Check the Autogain area box if the Autogain checkbox was selected in the Parameter Control (Figure 13).
4. Check the Single area box option. This is the preferred option when scanning single and multiplex NimbleGen arrays.
5. Review and if necessary adjust the scan area:

   The scan area determines the region of the slide that will be scanned. It should be large enough to capture the entire array area on the slide.

   - For NimbleGen 2.1M and 3x720K arrays, use the default selection for the scan area.
   - For NimbleGen 385K and 4x72K arrays, reduce the scan area, which will reduce the scan time. Scan area files specific for the NimbleGen 385k or 4x72K array are available for download at *www.nimblegen.com/scanner/* under *Download*.

   To use scan area(s) provided from NimbleGen

   a. Click **Load**.
   b. Select and open the area file.
   c. Click **Apply** to confirm your settings.

   To manually define and adjust scan area

   a. Capture the entire designated feature area. Move the mouse pointer inside the white scan area rectangle and click to enable to move cursor.
   b. Drag the rectangle over the desired feature region.
   c. If necessary, adjust the size of the rectangle by dragging a corner/side handle.
   d. Click **Apply** to confirm your settings.
   e. If desired, click Save to save settings to an area file for future use.

6. Review and if necessary adjust the autogain area.

   Ensure that the autogain area is located over features when determining PMT gain values automatically. Do not place the autogain area offset between or outside of the feature area to avoid incorrect PMT gain values.

   a. Position the mouse pointer inside the respective rectangle and click to enable the move cursor.
   b. Drag the rectangle to the desired location ensuring the rectangle is approximately 10mm from the edge of the default scan area.
   c. Size the rectangle by dragging the side and corner handles.
   d. Figure 15 provides guidance as to the size of and where to locate the autogain area.
   e. Click **Apply** to confirm your settings.

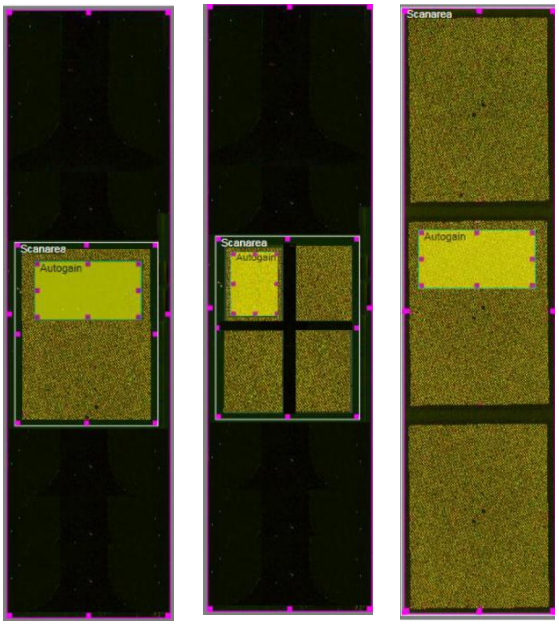


Figure 15: Examples of Autogain Areas for NimbleGen Arrays

7. Set the parameters for scanning another slide

   a. Repeat the instructions above for each slide.
   b. Use one of the following options to set parameters for another slide.
      - Copy and paste the parameter settings by right-clicking the slide in the magazine control table and selecting **Copy**. Then right-click the other slides to be scanned and select **Paste**.
      - If you saved parameter file(s) and area file(s), click **Load** in the Parameter Control or Area Definition Control, respectively, to select a file and then click **Apply** to confirm your selection.

Step 4. Scanning the Slides

1. Click the **Start/Stop Scan** button ( ) in the Scan Control (Figure 16).
2. Specify the folder and file name to save the session file. The scanning process should begin.
3. Once scanning begins, the Parameter Control and Area Definition Control close. The Image View, Slide View, and Spot View open (Figure 16). The current task is identified in the progress bar.

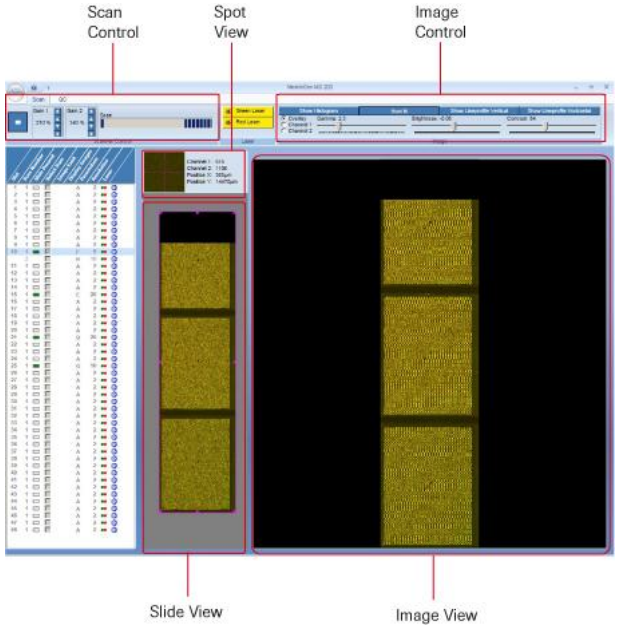


Figure 16: Example of a Run Time Window

4. *Optional:* To manually adjust the PMT gain while scanning at 5 µm or higher resolution, refer to the NimbleGen MS 200 Microarray Scanner Operator's Manual.

Notes

Chapter 6. Extracting Data with DEVA Software

Chapter 6 describes the general steps involved in importing a scanned image and extracting the data using DEVA software (version 1.0.2 or later). Refer to the *DEVA Software User's Guide* for computer system requirements and detailed information on using the software.

When analyzing 2.1M images or any images scanned at 2 µ resolution, you must use a computer with a 64 bit operating system, and at least 8 gigabytes of RAM.

Setting up a Project

To begin the automatically extracting data from your images using DEVA software, you must set up a new project according to instructions in Chapter 3 of the *DEVA Software User's Guide*.

Selecting Analysis Workflows

DEVA software will run analyses on the data from your bursted and aligned images using the specified application type: ChIP-chip or DNA Methylation.

Chapter 4 of the *DEVA Software User's Guide* provides information on how to:

- Select a workflow
- Use the *Analysis Workflow* window
- View examples of workflows
- Create customized workflows for your applications.

You can set up these application-specific workflows to run automatically each time a new image file is added to your image directory. For each application type, you can select one or more workflows that DEVA software will run automatically.

Loading Designs

DEVA software analyzes your scanned array images using the correct design files by matching the barcode on the design file(s) to the barcodes on the scanned image(s). After initially uploading the design (.zip) file, the design will continue to be used for new images, as long as the barcodes match and a default workflow analysis has been selected.

Chapter 5 of the *DEVA User's Guide* provides information on how to load design files and add a design file to a slide barcode.

Importing Annotation

Many annotation files will load automatically as part of the NimbleGen Array design (.zip) file. However, if other annotation files are needed, you can load them manually.

Chapter 6 of the *DEVA Software User's Guide* provides information on how to select, load, and view annotations.

Monitoring Processing of Images

DEVA software will automatically upload and process images that are placed in the Image Directory. The *DEVA Software User's Guide* provides information on how to monitor the processing of images.

Reviewing Processed Slides

Chapter 8 of the *DEVA Software User's Guide* provides information on how to view all of the slides that have been uploaded by DEVA software that have been successfully burst (if multiplex) and aligned.

Roche NimbleGen recommends a manual review of the thumbnails and/or entire image to confirm correct alignment regardless of the alignment score value.

Adding Information in the Hybridization Plan

The Hybridization Plan is useful for recording details about your array experiment. You can create a plan in DEVA software after you have imported a design file.

Chapter 7 of the *DEVA Software User's Guide* provides information on how create a hybridization plan.

Reviewing Experimental Results

DEVA software will automatically run the default analyses for each application. When the jobs have completed, you can view the results. Note that the workflows in DEVA software do not include the generation of the promoter reports that map peak data to annotation files.

Chapter 9 of the *DEVA Software User's Guide* provides information on how to access and view the analysis results.

Mapping Peaks

The workflows in DEVA software do not include the generation of the promoter reports that map peak data to annotation files.

Chapter 12 of the *DEVA Software User's Guide* provides information on how to generate these promoter reports.

Confirming Experimental Integrity

As noted in this guide, you should add a unique STC to each experimental (IP) and control (input) sample pair prior to loading onto multiplex arrays as described in Chapter 4, Step 1. This control hybridizes to probes on the microarray and enables you to confirm the sample identity on each array and ensure integrity of the experiment. STC probes are placed as repeating sets of 20 along the perimeter of each array and as two 4 x 5 blocks in the upper left corner and in the center of the array (Figure 17).

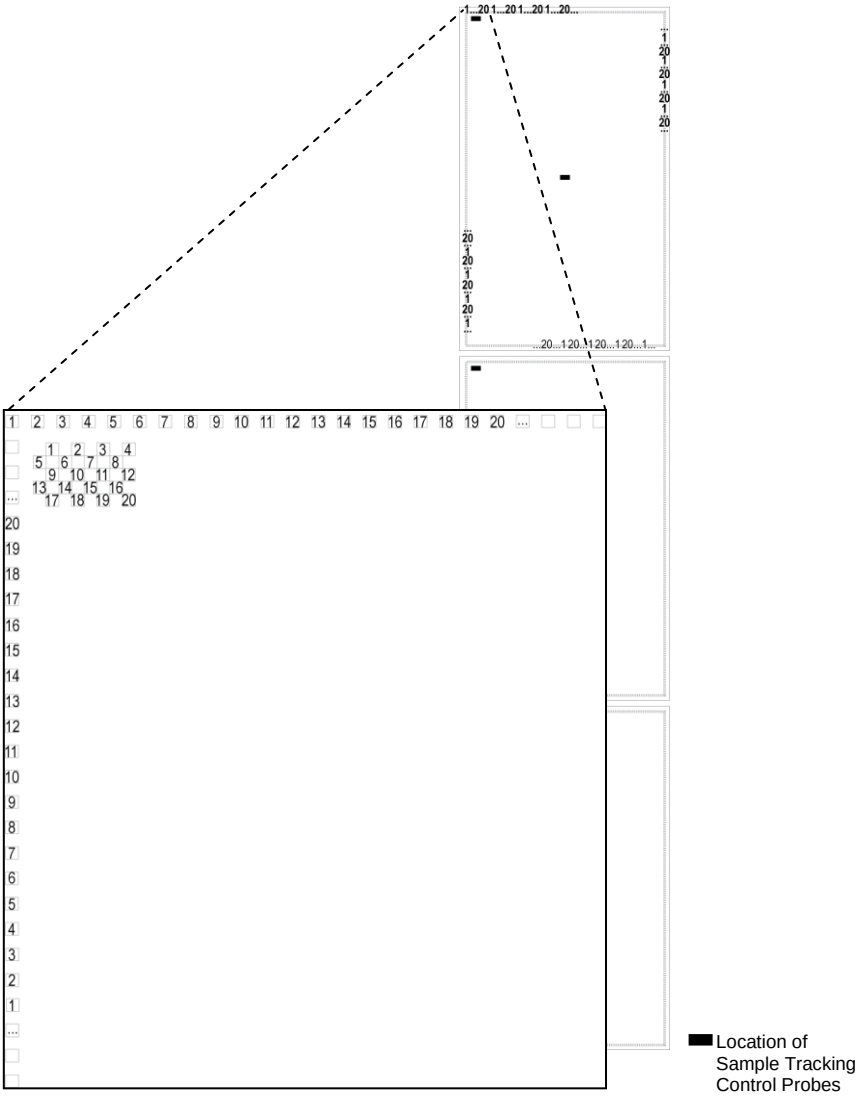


Figure 17: Location and Numbering of Sample Tracking Control Probes on a 3x720K Array

Roche NimbleGen recommends performing a sample tracking analysis and visually checking the STC features along the perimeter to confirm that the correct sample has been added to each array. The sample tracking analysis can be done by generating a Sample Tracking Control Report. Visually checking the STC features along the perimeter can be done using the following steps:

1. From the *Processed Data Slides* tab, select the slide barcode of interest.
2. Click **Alignment Details**.
3. Select the desired image.
4. Click **Adjust Alignment**.
5. If necessary, zoom into the upper left corner of the array with the zoom tool.
6. Locate the repeating set of 20 features along the perimeter of the array. **Figure 18** below shows an example of an array hybridized with a sample containing STC 1.

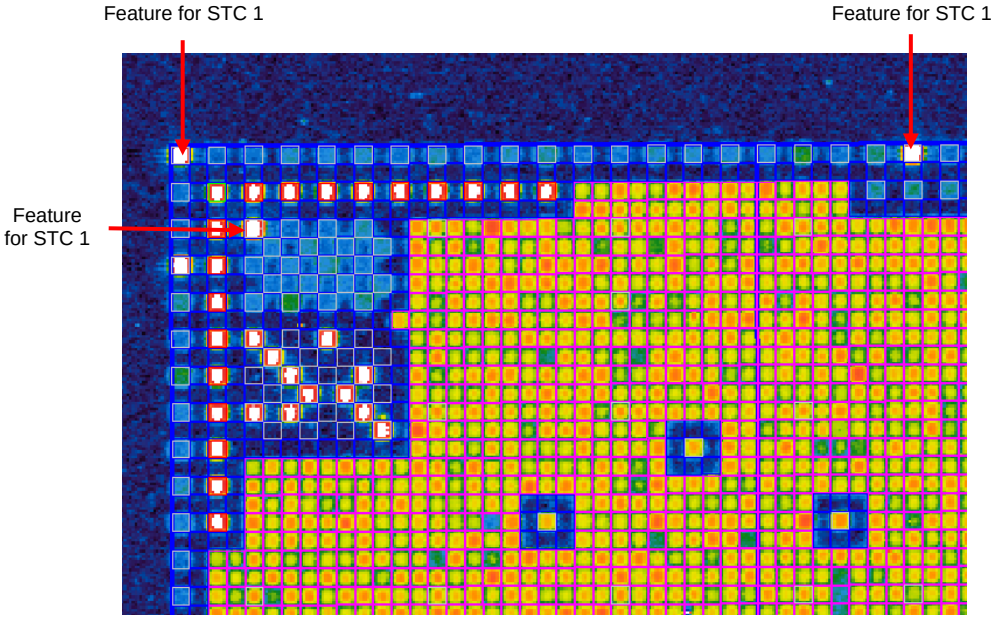


Figure 18: Example of an Array Hybridized with Sample Containing STC 1

7. Survey the entire perimeter of the array. If more than one STC is visible, the integrity of your data could be compromised due to cross-contamination that occurred during sample preparation, loading, or hybridization. Roche NimbleGen recommends that you repeat experiments that indicate cross-contamination.

Exporting Reports

After you have examined your analysis in the *Analysis Results* window, you may want to export reports, data or pictures for use in other applications.

See the *DEVA Software User's Guide* for descriptions of the over 20 reports that you can generate from your data. Two of the most commonly generated reports are described below.

Sample Tracking Control Report

The Sample Tracking Control report is based on signal intensities of features in the two 4 x 5 blocks with Sample Tracking Control probes (**Figure 18**). Using this report and sample records from your experiment, you can confirm the intended sample was hybridized to the intended array.

Experimental Metrics Report

The Experimental Metrics report provides a set of metrics that can be used to establish guidelines for determining the quality of the data. Roche NimbleGen recommends that you use these metrics to develop criteria for assessing the overall quality of your microarray experiments. The metrics provided will vary according to application, array format, probe design, organism, sample type and quality, and hybridization conditions. Refer to the *Guide to Interpreting the Experimental Metrics Report* for more details on the metrics listed in this report.

Learn More about Your ChIP-chip Data

Signal Intensity (Raw) Data

Signal intensity data is extracted from the scanned images of each array using DEVA software. Signal intensities for each probe are saved in pair files (.txt), the raw data format for ChIP-chip experiments. View pair files using a text editor. (Refer to "Step 6. Create Pair Reports" on page 44 for information on how to create pair files.)

Scaled Log_2 Ratio Data

Each feature on the array has a corresponding scaled log_2 ratio, which is the ratio of the input signals for the experimental (IP) and control (input) samples co-hybridized to the array. The log_2 ratio is computed and scaled to center the ratio data around zero. Centering is performed by subtracting the bi-weight mean for the log_2 ratio values for all features on the array from each log_2 ratio value.

Peak Data

Using DEVA software, peak data files (.gff) are generated from the scaled log_2 ratio data. DEVA software detects peaks by searching for 4 or more probes whose signals are above the specified cutoff values, ranging from 90% to 15%, using a 500 bp sliding window. The cutoff values are a percentage of a hypothetical maximum, which is the mean + 6 [standard deviation]. The ratio data is then randomized 20 times to evaluate the probability of "false positives." Each peak is then assigned a false discovery rate (FDR) score based on the randomization. In general, use these guidelines when reviewing FDR scores:

- The lower the FDR score, the more likely the peak corresponds to a protein binding site.
- For most data sets, peaks with FDR score ≤ 0.05 very often represent the highest-confidence protein binding site(s).
- Peaks with FDR score between 0.05 and 0.2 are also indicative of a binding site.
- Peaks with FDR score > 0.2 are generally not considered high-confidence binding sites.

Map Peaks Reports

Once you identify peaks in your data, you can determine where these peaks lie in the genome relative to genes or other relevant annotations. The analysis looks at the genomic locations of features and associates those features with genomic annotation to create a summary of what genes are associated with the detected peaks. To run this analysis, you need the GFF file containing the detected features and the genome annotation GFF file provided with your microarray.

You can view the map peaks reports using spreadsheet software, such as Microsoft Excel:

- **All pairs of track data points overlapping/nearby features:** Generates a report that maps every combination of data points and nearby or overlapping annotation feature.
- **Nearest track data point (if any) to each feature:** Generates a report containing the nearest data point that maps to a nearby or overlapping annotation feature.
- **Nearest feature (if any) to each track data point:** Generates a report containing the nearest feature that maps to a nearby or overlapping track data point.

To effectively analyze peak data, you should sort the data in promoter reports according to FDR, peak score, gene name, chromosome, distance to TSS, etc.

The table below identifies the fields on the promoter reports (.xls):

Field	Description
DATA_INDEX	An ID for each mapped data point.
CHROMOSOME	Chromosome associated with the data point.
DATA_START	First base of the data point on the chromosome.
DATA_END	Last base of the data point on the chromosome.
PEAK_SCORE	The $\log_2$ ratio of the fourth highest probe in the peak.
Various	The Plot Name for the track (data file) selected to generate the Map Peaks report. For example, using DEAV, if the Peak Results track is used the header of this column will be Peaks and the value contained in the column will be the $\log_2$ ratio of the fourth highest probe in the peak.
Various	The Valued Attributes of the track selected to generate the Map Peaks report. For example, using DEVA, if the Peak Results track is used the header of this column will be FDR and the value of the column will be the FDR for the peak.
FEATURE_TRACK	The annotation track against which peaks were mapped; it is the transcription start site for promoter reports.
FEATURE_STRAND	Strand of the transcript.
FEATURE_START	First base of the feature on the chromosome. ***Note:*** *For the transcription start site, feature size is 1; therefore, start and end positions are the same.*
FEATURE_END	Last base of the feature on the chromosome. ***Note:*** *For the transcription start site, feature size is 1; therefore, start and end positions are the same.*
CENTER_TO_CENTER_DISTANCE FEATURE_TO_DATA_POINT	Center-to-center distance of data point to feature.
SHORTEST_DISTANCE_FROM_ FEATURE_TO_DATA_POINT	Shortest distance between data point and feature. This value will be zero if the data point and feature overlap.
Name	Gene symbol of the transcript.
accession	GenBank accession number of the transcript.
description	Full gene name of the transcript.
ncbi_gene_id	NCBI Entrez GeneID of the transcript.
synonyms	Other alias symbol(s) of the transcript.
Parent	The internal identification number of the transcript from which this transcription start site is generated.

Data Provided with Custom Designs

If your array design was customized, some of the files described above may not be provided. For instance, annotation files (.gff) may not be readily available for less common genomes, which will result in no promoter reports being generated. Also, if a positions file (.pos) is not available (because genomic coordinates were not provided for a custom design), no ratio files (.gff), peak data files (.gff), or promoter reports (.xls) are generated.

Chapter 7. Troubleshooting

Chapter 7 helps you troubleshoot problems that occurred with your microarray experiment.

Sample Quality

Problem	Possible Cause	Recommended Corrective Action
260/230 Absorption Ratio is less than 1.6.	DNA sample is contaminated with carbohydrate or phenol/chloroform.	Clean up samples using a DNA cleanup column or phenol/chloroform extraction followed by ethanol precipitation.
260/280 Absorption Ratio is less than 1.7.	DNA sample is contaminated with protein.	Clean up samples using a DNA cleanup column or phenol/chloroform extraction followed by ethanol precipitation.
260/280 Absorption Ratio is greater than 2.0.	DNA sample is contaminated or degraded.	Clean up samples using a DNA cleanup column or phenol/chloroform extraction followed by ethanol precipitation. Or if degraded, repeat the experiment.

Labeling

Problem	Possible Cause(s)	Recommended Corrective Action
Labeling yield is less than 34 µg per reaction (or less than 24 µg total for 2.1M arrays).	DNA sample is contaminated or degraded.	Check absorption ratios and gel image. If necessary, clean up samples or repeat the ChIP protocol. Repeat labeling.
	Primers were not diluted correctly. ß-Mercaptoethanol was not added. Diluted primers are older than 4 months.	Prepare a fresh dilution of nonamer primers, ensuring that the ß-Mercaptoethanol is fresh (opened less than 6 months). Repeat labeling.
	Klenow enzyme is expired or degraded.	Check the expiration date and follow the labeling kit's storage requirements. Repeat labeling, using fresh enzyme, if necessary.
	Primers are degraded.	Store primers at -15 to -25°C, protected from light, and avoid freeze-thaw cycles. Repeat labeling, using fresh primers, if necessary.
	dNTPs are expired or degraded.	Check the expiration date, follow the labeling kit's storage requirements, and avoid freeze-thaw cycles. Repeat labeling, using fresh dNTPs, if necessary.

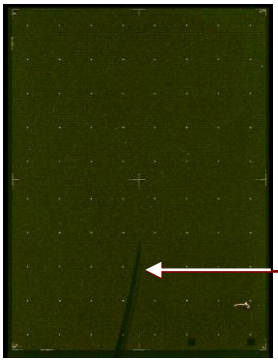
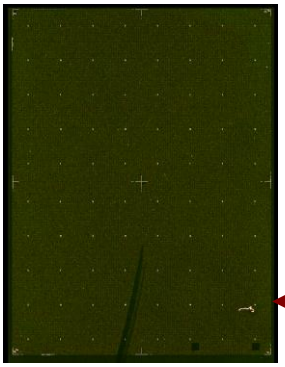
Problem	Possible Cause(s)	Recommended Corrective Action
Pellets are not solid.	Incorrect ratio of reaction volume to isopropanol.	Repeat labeling, making sure to precipitate samples with 110 µl of isopropanol per reaction.

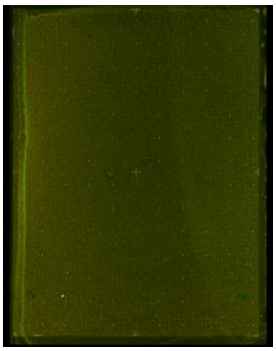
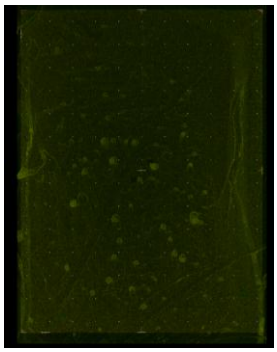
Note: *You can run a positive control reaction using Roche Human Genomic DNA (Catalog No. 11 691 112 001), following the labeling protocol described in Chapter 3. Sample Labeling.*

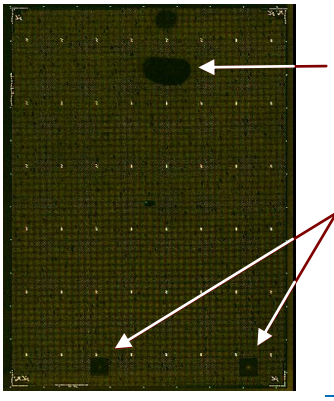
Hybridization

Problem	Possible Cause	Recommended Corrective Action
Mixer is poorly aligned on slide.	The slide was not flush in the PMAT.	Remove the mixer using the Mixer Disassembly Tool then reassemble using a new mixer.
Hybridization solution does not enter the hybridization chamber.	The pipette tip is not situated properly on the fill port.	Refer to page 28 for proper loading technique. Ensure that the pipette tip is placed firm and snug against the fill port before dispensing.
Bubbles formed when loading the sample into the mixer's hybridization chamber.	Air was present in the pipette tip.	Use Gilson Positive Displacement Pipettes and follow the instructions on page 28 for proper loading technique. Using the pipette, remove the bubbles and replace with hybridization solution. Remove the bubbles or push them to the corners using the mixer brayer.
Sample leaked out of the mixer before or during the hybridization.	The mixer was not fully adhered to the slide due to incomplete braying.	Refer to page 26 for proper braying technique. Repeat hybridization.
	The mixer port seals/multi-port seals were not fully adhered to the mixer.	Refer to page 28 for proper sealing technique. Ensure that excess hybridization solution has been wiped from the ports before adhering the mixer port seal/ multi-port seal. Repeat hybridization.

Scanning

Problem	Possible Cause	Recommended Corrective Action
Scratches and/or fingerprints are seen on the array image.	The slide was mishandled or dropped.	Grip the slide only on its edges and handle with care. Wear gloves when handling slides.
		
Dust is seen on the array image.	The array was exposed to environmental dust.	If the amount of dust present is small, use a compressed gas nozzle to gently blow compressed nitrogen or argon gas across the array to remove the dust. Rescan the array. If the amount of dust is excessive, repeat all wash steps, dry, and scan.
		
	Dust or dirt was present in the microarray dryer.	Clean the microarray dryer as instructed in its operator's manual. Repeat all wash steps, dry, and scan.

Problem	Possible Cause	Recommended Corrective Action
Wash artifacts are seen on the array image. 	Slides were not washed and dried completely. 	Use the dryer that meets recommended specifications . Repeat the slide wash, dry, and scan steps, making sure to transfer the slide immediately from the wash solution to the microarray dryer. Blot residual wash buffer from the edges of the slide. Make fresh wash buffers for each batch of slides.
Bright streaks are seen on the array image.	The microarray dryer does not accelerate fast enough.	Ensure proper function and maintenance of the microarray dryer.
Part of the array is missing from the array image.	The scan area is not specified properly.	Refer to page the MS200 User's Guide for instructions on how to specify the scan area. Repeat the scan ensuring that fiducial features are included in the scan area.
The array image appears dim.	Gain or PMT settings are not adjusted correctly.	Refer to the MS200 User's Guide for instructions on how to adjust gain or PMT settings. Repeat scan.
	Hybridization and/or wash conditions are too stringent.	Check that the hybridization solution was prepared correctly (refer to page 24) and the NimbleGen Hybridization System is set to and maintaining +42°C. Repeat hybridization. Refer to page 29 for proper washing technique. Repeat hybridization.
	Sample leaked out of the mixer during hybridization due to incomplete braying.	Refer to page 26 for proper braying technique. Repeat hybridization.
	Cy dye(s) are degraded due to exposure to light, ozone, and/or humidity.	Store primers at -15 to -25°C, protected from light. Maintain ozone levels below 5 ppb and humidity levels below 40%. Repeat hybridization.
The fiducial features appear dim or blank.	The alignment oligo was either not added to the hybridization solution or was degraded due to repeated freeze-thaw cycles.	Repeat hybridization, using fresh alignment oligo, if necessary.

Problem	Possible Cause	Recommended Corrective Action
Features appear blank on portions of the array.	The slide contains a scratch or fingerprint.	Grip the slide only on its edges and handle with care. Wear gloves when handling slides. Repeat hybridization.
	One or more bubbles were present in the hybridization chamber.	Repeat hybridization if blank regions cover greater than 5% of the array area.
		
	Wash buffer dried onto the array surface in between wash steps.	Ensure that slides are transferred quickly between wash steps. Repeat hybridization.
The array image is too bright.	Hybridization and/or wash conditions are not stringent enough.	Check that the hybridization solution was prepared correctly (refer to page 24) and the NimbleGen Hybridization System power is on, mixing is on, and temperature is set to and maintaining +42°C. Repeat hybridization. Refer to page 29 for proper washing technique. Repeat hybridization.
	Gain or PMT settings are not adjusted correctly.	Refer to for the MS200 User's Guide for instructions on how to adjust gain or PMT settings. Repeat scan.

Problem	Possible Cause	Recommended Corrective Action
The array image brightness is uneven.	One or more bubbles were present in the hybridization chamber, and/or there was poor mixing during hybridization.	Repeat hybridization. If the problem persists, refer to the *NimbleGen Hybridization System User's Guide* for troubleshooting information.
	Sample leaked out of the mixer during hybridization due to incomplete braying	Refer to page 26 for proper braying technique. Repeat hybridization.
	The sample pellet was not properly rehydrated or mixed with the hybridization solution.	During sample preparation, be sure to vortex and spin the sample before and after the +95°C incubation. Repeat hybridization.
	Array washing was not done properly.	Refer to 29 for proper washing technique. Repeat hybridization.
The array image is uniformly yellow and lacks red or green saturated features.	Hybridization and/or wash conditions are not stringent enough.	Check that the hybridization solution was prepared correctly (refer to page 24) and the NimbleGen Hybridization System is set to and maintaining +42°C. Repeat hybridization. Refer to page 29 for proper washing technique. Repeat hybridization.
The array image is either too green or too red.	Gain or PMT settings are not adjusted correctly.	Refer to the MS200 User's Guide for instructions on how to adjust gain or PMT settings. Repeat scan.
	Experimental (IP) and control (input) samples were not added in equal amounts.	Combined sample pellets and hybridization solution should be violet in color; pink or blue color suggests that too much of one sample was added.

Sample Tracking Controls (STCs)

Problem	Possible Cause	Recommended Corrective Action
STC features are not visible in the STC control regions that are located along the perimeter, in the upper left corner, and in the center of the array.	Experimental (IP) and control (input) samples were not resuspended in an STC, or the STC was degraded from repeated freeze-thaw cycles.	Repeat hybridization, using a fresh STC, if necessary.
STC features representing multiple STCs are visible on the array image or reported in the Sample Tracking report.	Sample integrity was compromised during sample preparation, loading, or hybridization.	Repeat hybridization, ensuring the following: ■ The mixer is fully adhered to the slide before loading sample. Refer to page 26 for proper braying technique. ■ Excess sample is removed from around the loading ports. Refer to page 28 for proper sealing technique. ■ The mixer port seals/multi-port seals were fully adhered to the mixer after loading sample. Refer to page 28 for proper sealing technique.

Data Analysis

Problem	Possible Cause	Recommended Corrective Action
Data appear noisy.	Experimental (IP) and control (input) samples were of poor quality.	Refer to to *Chapter 2. Preparing Samples and Performing QC* (page 15). Repeat labeling and hybridization.
	Species/strain variation between experimental (IP) and control (input) samples.	If experimental (IP) and control (input) samples are of different species or strains, data may be noisy.
No peaks are detected.	Hybridization and/or wash conditions are not stringent enough.	Check that the hybridization solution was prepared correctly (refer to page 24) and the NimbleGen Hybridization System is set to and maintaining +42°C. Repeat hybridization. Refer to page 29 for proper washing technique. Repeat hybridization.
	Experimental (IP) sample was not significantly enriched.	Check enrichment level of experimental (IP) sample compared to control (input) sample.
Peaks have low confidence values.	Experimental (IP) sample was not significantly enriched.	Check enrichment level of experimental (IP) sample compared to control (input) sample.
The $\log_2$ ratio values are muted.	The slide-mixer assembly reached +15 to +25°C between the hybridization and wash steps.	Remove the slide-mixer assemblies one at a time from the NimbleGen Hybridization System, immediately disassemble, and place in Wash Buffer I.
	Experimental (IP) sample was of poor quality.	Refer to to *Chapter 2. Preparing Samples and Performing QC* (page 15). Repeat labeling and hybridization.
	Hybridization and/or wash conditions are not stringent enough.	Check that the hybridization solution was prepared correctly (refer to page 24) and the NimbleGen Hybridization System is set to and maintaining +42°C. Repeat hybridization. Refer to page 29 for proper washing technique. Repeat hybridization.

Appendix A. Limited Warranty

ROCHE NIMBLEGEN, INC.
NIMBLEGEN ARRAYS

1. Limited Warranty

A. Products: Roche NimbleGen, Inc. ("Roche NimbleGen") warrants that its Products conform to its published specifications and are free from defects in material or workmanship. Customer's sole and exclusive remedy (and Roche NimbleGen's sole and exclusive liability) under this limited warranty shall be to either (a) replace the defective Products, or (b) provide Customer with a refund, as solely determined by Roche NimbleGen.

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If you have any questions concerning service of this product, please contact your local Roche Microarray Technical Support. Go to *www.nimblegen.com/arraysupport* for contact information.

Evidence of original purchase is required. It is important to save your sales receipt or packaging slip to verify purchase.

Notes

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