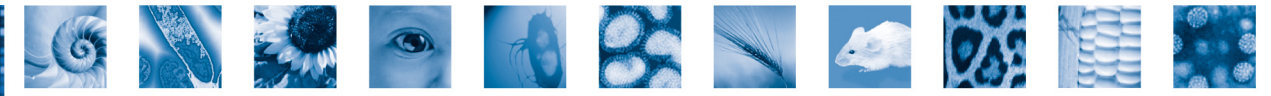
NimbleGen Array User's Guide
DNA Methylation Arrays

Version 7.1



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

Editions

Version 1.0, 16 Jan 2007; Version 2.0, 7 Sep 2007; Version 3.0, 25 Jun 2008; Version 4.0, 27 Oct 2008; Version 5.0, 11 Mar 2009; Version 6.0, 21 Oct 2009; Version 7.0, 21 Dec 2009; Version 7.1, 28 May 2010

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Chapter 1. Before You Begin

This *User's Guide* describes the process for hybridization of samples prepared by methylated DNA immunoprecipitation (MeDIP) (and amplified by whole genome amplification [WGA] if necessary) on these NimbleGen arrays formats:

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

The first part of the process requires quality control of experimental (IP) and control (input) samples to verify quality prior to microarray hybridization. You then independently label these samples using a NimbleGen Dual-Color DNA Labeling Kit and co-hybridize them to a NimbleGen DNA Methylation array using a NimbleGen Hybridization System. Following hybridization, the arrays are washed, dried, and scanned using a NimbleGen MS 200 Microarray Scanner. Array data are extracted and analyzed using our NimbleScan software and SignalMap software.

Example procedures for dsDNA digestion, immunoprecipitation, and amplification (WGA) are described in the MeDIP protocol, available from Roche Microarray Technical Support. Refer to page 8 for contact information.

What's New?

Version 7.1 of this *User's Guide* includes instructions on using NimbleScan v2.6 Software for DNA methylation experiments.

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/*.

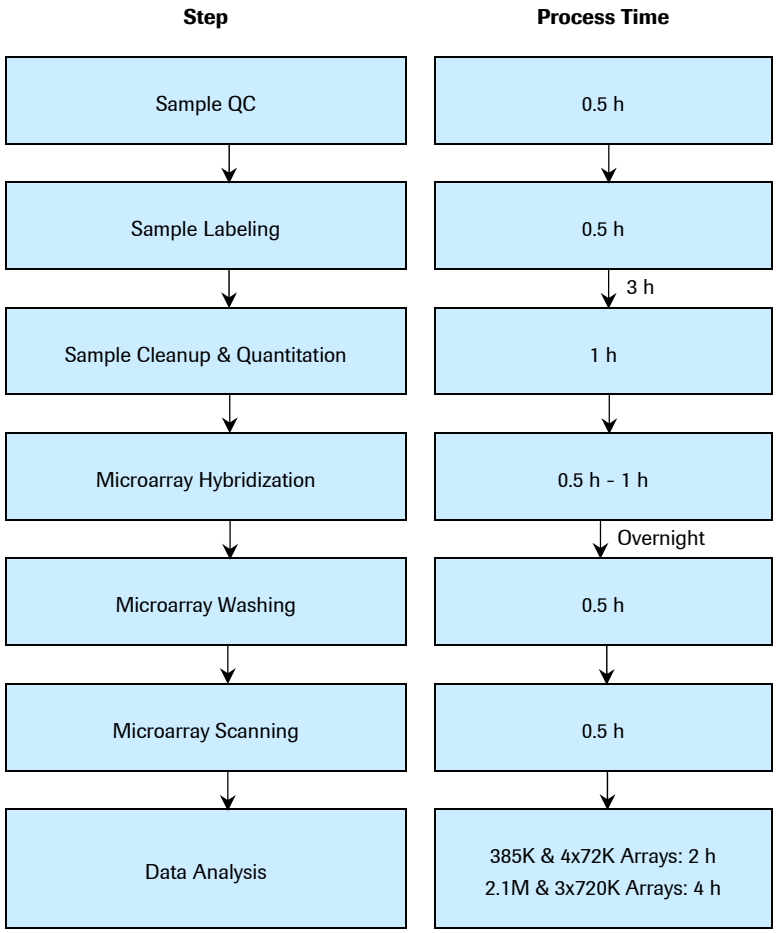


Figure 1: Workflow for NimbleGen DNA Methylation Arrays. Steps in the process and estimated time for each step, based on the processing of one slide, are shown in the boxes. Incubation times are indicated beneath the appropriate process times.

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 room temperature for use by the expiration date. Once the seal is broken, store NimbleGen arrays in a desiccator at room temperature until use.

Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- 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.
- Cy dyes are ozone sensitive. Take the necessary precautions to keep atmospheric ozone levels below 20 ppb (parts per billion).
- Cy dyes are humidity sensitive. Take the necessary precautions to keep humidity levels below 40%.
- 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 room temperature 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

Choose between 4- or 12-bay NimbleGen Hybridization Systems.

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 Hybridization System 12*	Roche NimbleGen	12 slides	05 223 687 001 (110V)
			05 223 695 001 (220V)
NimbleGen Microarray Dryer	Roche NimbleGen	24 slides	05 223 636 001 (110V)
			05 223 644 001 (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.
NimbleScan v2.6	Roche NimbleGen	05 933 315 001 (Individual License)
		05 933 331 001 (Site License)
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
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)	5 mixers	05 391 717 001
	10 mixers	05 223 725 001
NimbleGen X4 Mixer (for 4x72K arrays; includes mixer multi-port seals)	5 mixers	05 391 725 001
	10 mixers	05 223 733 001
NimbleGen HX1 Mixer (for 2.1M arrays; includes mixer port seals)	5 mixers	05 391 733 001
	10 mixers	05 223 741 001
NimbleGen HX3 Mixer (for 3x720K arrays; includes mixer port seals)	5 mixers	05 391 741 001
	10 mixers	05 223 750 001

Component	Package Size / Process Quantity	Catalog No.
NimbleGen Dual-Color DNA Labeling Kit Contents: ■ Nuclease-free Water (2 x vial 1) ■ Random Primer Buffer (vial 2) ■ Cy3-Random Nonamers (vial 3) ■ Cy5-Random Nonamers (vial 4) ■ Klenow Fragment (3'->5' exo-) 50 U/µl (vial 5) ■ 10 mM dNTP Mix (vial 6) ■ Stop Solution (0.5 M EDTA) (vial 7) ■ 5 M NaCl (vial 8)	10 Cy3 labeling reactions and 10 Cy5 labeling reactions	05 223 547 001
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

Component	Package Size / Process Quantity	Catalog No.
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 aerosol compressed air for this purpose is not recommended and could compromise array and data quality.

Technical Support

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

Conventions Used in This Manual

Text Conventions

To impart information that is consistent and memorable, the following text conventions are used in this *User's Guide*:

Convention	Description
Numbered listing	Steps in a procedure that must be performed in the order listed.
Italic type, blue	Points to a different chapter in this *User's Guide* to consult or to a web site.
Italic type	Identifies the names of controls (checkboxes, option buttons, etc.) in dialog boxes, windows, or message boxes in software.
Bold type	Identifies buttons and menu names when operating software.
Underscore and brackets	A placeholder for information such as in the actual name of a directory in a path is enclosed in brackets, e.g. <install path>. Placeholders (for file names, numbers, dates, etc.) are separated by an underscore (_), e.g. <Barcode>_<User Text>_<Laser WL>.

Symbols

The following types of notices may be used in this manual to highlight important information or to warn the operator of a potentially dangerous situation:

Symbol	Description
	Important Note. Used to bring your attention to important annotation.
	Information Note: Designates a note that provides additional information concerning the current topic or procedure.

Notes

Chapter 2. Sample Preparation & QC

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

Sample Requirements

- 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.
- 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).

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

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	3.5 µg	1.5 µg
Control (Input) Sample	1.5 µg	1.5 µg	3.5 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 nuclease-free water or 1X TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 7.5 - 8.0).
  - An $A_{260}/A_{280} \geq 1.7$ and $A_{260}/A_{230} \geq 1.6$.

Sample QC

1. Transfer 200 ng of each sample to a sterile microcentrifuge tube. Store the remainder of your sample set at -20°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 be carried through labeling and hybridization due to the risk of poor results.

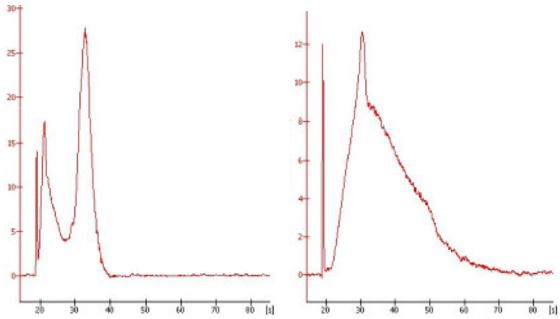


Figure 2: Examples of Bioanalyzer Traces of Nondegraded Samples

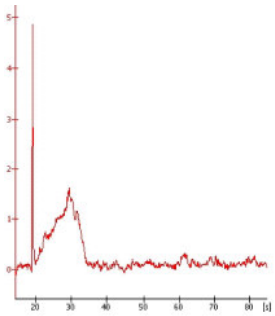


Figure 3: Example of Bioanalyzer Trace of a Degraded Sample

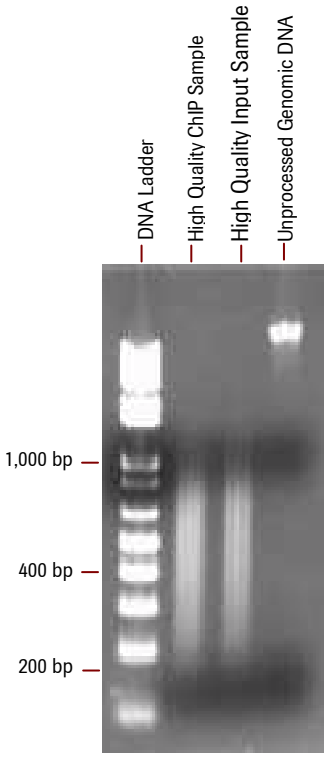


Figure 4: Example of Agarose Gel Electrophoresis for Samples of Good Quality

Chapter 3. Sample Labeling

Chapter 3 describes how to label your experimental (IP) and control (input) samples using a NimbleGen Dual-Color DNA Labeling Kit. Be aware of the following when using the NimbleGen Dual-Color Labeling Kit:

- Aliquot dNTPs and Cy primers into single-use amounts.
- 5 M NaCl 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.

- **Control (input) sample:** A small aliquot of the starting DNA is carried through the labeling protocol and used as a reference sample.
- **Non-Specific Binding (NSB) sample:** 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.

1. Prepare the following solution in a 1.5 ml microfuge tube:

Random Primer Buffer	All Array Formats	Notes
Random Primer Buffer (vial 2)	998.25 µl	Prepare fresh buffer each time primers are resuspended.
β-Mercaptoethanol*	1.75 µl	
Total	**1 ml**	

* 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 462 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 -20°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	385K, 4x72K, and 3x720K Arrays		2.1M Arrays	
	Experimental (IP) Sample	Control (Input) Sample	Experimental (IP) Sample	Control (Input) Sample
Sample prepared in Chapter 2	1 µg	1 µg	1 µg (in each of 3 tubes)*	1 µg (in each of 3 tubes)*
Diluted Cy3-Random Nonamers from step 2		40 µl		40 µl (per tube)
Diluted Cy5-Random Nonamers from step 2	40 µl		40 µl (per tube)	
Nuclease-free Water (vial 1)	To volume (80 µl)	To volume (80 µl)	To volume (80 µl) (per tube)	To volume (80 µl) (per tube)
Total	**80 µl**	**80 µl**	**80 µl (per tube)**	**80 µl (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 0.2 ml thin-walled PCR tubes with each containing 1 µg 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. If labeled DNA yields are consistently high, the number of labeling reactions for a 2.1M array can be reduced from 3 to 2 reactions (34 µg labeled DNA per sample is required for each array).

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
10 mM dNTP Mix (vial 6)	10 µl
Nuclease-free 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 (0.5 M EDTA).

Component	All Array Formats	
	Experimental (IP) Sample	Control (Input) Sample
Reaction volume from step 6	100 µl	100 µl
Stop Solution (0.5 M EDTA) (vial 7)	10 µl	10 µl
Total	**110 µl**	**110 µl**

11. Add 5 M NaCl to each tube.

Component	All Array Formats	
	Experimental (IP) Sample	Control (Input) Sample
Reaction volume from step 10	110 µl	110 µl
5 M NaCl (vial 8)	11.5 µl	11.5 µl
Total	**121.5 µl**	**121.5 µl**

12. 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 11	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.

13. Vortex well. Incubate for 10 minutes at room temperature, protected from light.
14. 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.
15. Rinse pellet with 500 µl 80% ice-cold ethanol. Dislodge pellet from tube wall by pipetting a few times.
16. Centrifuge at 12,000 x g for 2 minutes. Remove supernatant with a pipette.
17. Dry contents in a DNA vacuum concentrator on low heat until dry (approximately 5 minutes), protected from light.
18. STOP POINT: Proceed to step 19, or store labeled samples at -20°C (up to 1 month), protected from light.
19. Spin tubes briefly prior to opening. Rehydrate each pellet in 25 µl Nuclease-free Water (vial 1) per reaction.
20. 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.
21. Quantitate each sample using the following formula:

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

    If using a NanoDrop Spectrophotometer, refer to the manufacturer's instructions to ensure accurate quantitation.
22. 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 3 labeling reactions, the hybridization can be performed with as little as 24 µg.

23. Dry contents in a DNA vacuum concentrator on low heat, protected from light.
24. STOP POINT: Proceed to *Chapter 4*, or store labeled samples at -20°C (up to 1 month), protected from light.

Chapter 4. Hybridization & Washing

Chapter 4 describes the NimbleGen protocol for sample hybridization and washing. Be aware of the following:

- The hybridization protocol requires a NimbleGen Hybridization System. Refer to its *User's Guide* for specific instructions on its use.
- The hybridization protocol requires adhering 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 -20°C. Protect tubes from light.

Step 1. Prepare Samples

1. Set the Hybridization System 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. Using components from a NimbleGen Hybridization Kit, 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**

5. 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**

6. 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.
7. 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. Prepare 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 aerosol 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. Gently blow compressed nitrogen or argon gas across the mixer and slide to remove dust.

   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. 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.

6. 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.

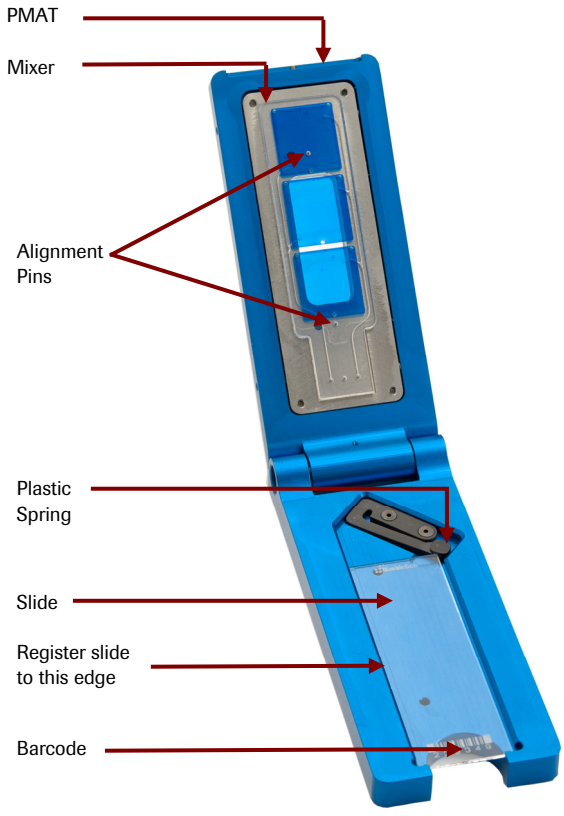


Figure 5: PMAT with HX3 Mixer and Slide. For photographic purposes only, blue coloring was used to show the location of the mixer's hybridization chambers. The hybridization chambers of the mixer you receive will not be blue.

7. Remove the mixer-slide assembly from the PMAT.
8. Place the mixer-slide assembly on the back of a 42°C heating block for 5 minutes to facilitate adhesion of the mixer to the slide.
9. 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 outwards. 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.
10. Place the mixer-slide assembly in the slide bay of the Hybridization System.

Step 3. Load & Hybridize Samples

1. Refer to the appropriate diagram below when loading sample:

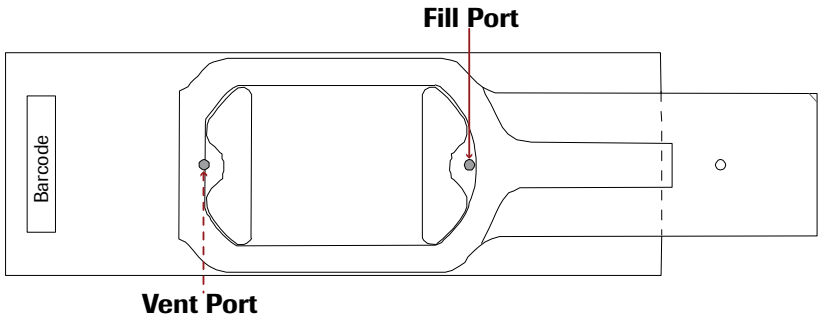


Figure 6: X1 Mixer and Slide for a 385K Array

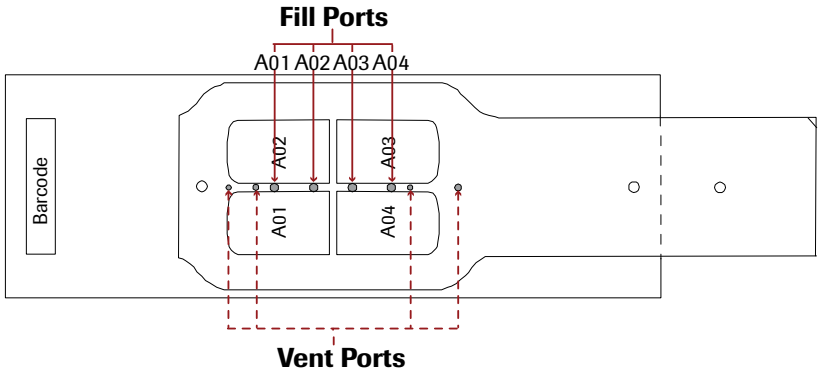


Figure 7: X4 Mixer and Slide for a 4x72K Array

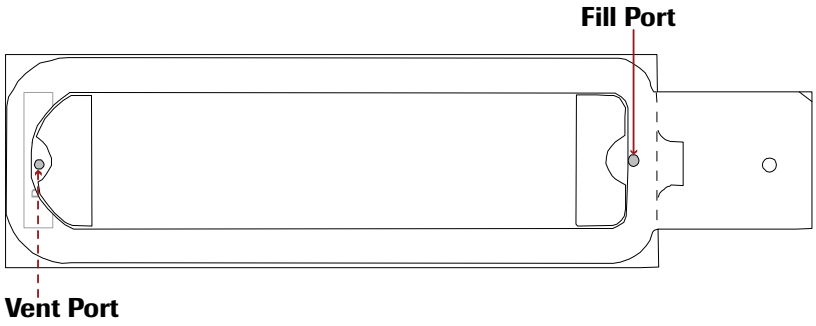


Figure 8: HX1 Mixer and Slide for a 2.1M Array

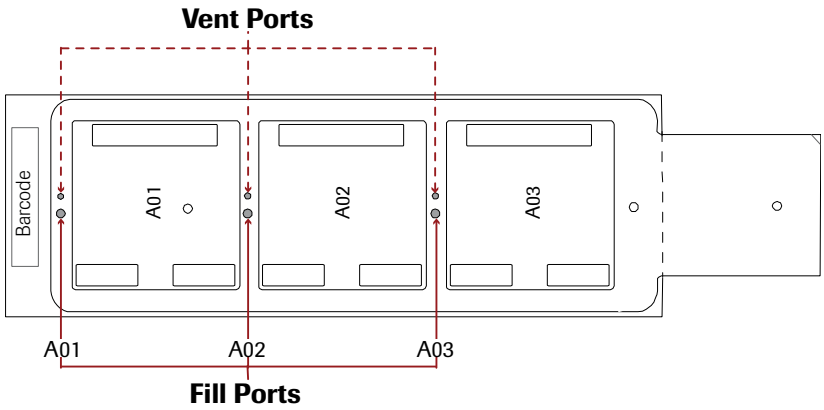


Figure 9: HX3 Mixer and Slide for a 3x720K Array

2. Keep the following in mind before loading sample:

   - Leave residual volume in the sample tube to avoid bubbles. The volumes listed in the table below account for this additional amount.
   - 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.

3. Using the appropriate Gilson Microman pipette, slowly 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 the 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. Use one mixer port seal to cover the fill port and another to cover the vent port on X1 or HX1 mixers. Use one mixer port seal to cover both the fill and vent ports on HX3 mixers, filling and sealing one chamber at a time. 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. Use one mixer multi-port seal to cover all fill and vent ports on X4 mixers. 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.
4. Close the bay clamp.
5. 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).
6. 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.
7. Hybridize sample at 42°C to the array(s) for 16 - 20 hours.

Step 4. Wash Hybridized Arrays

To ensure high quality data, it is important to proceed through all the washing and drying steps without interruption. The NimbleGen Microarray Dryer dries up to 24 slides at a time. 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 8).

   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°C 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. Roche NimbleGen recommends measuring the temperature of Wash I at every use. Keep the remaining three wash solutions at room temperature.
4. Insert the Mixer Disassembly Tool into the shallow dish containing warm Wash I. If you will be washing multiple slides, insert a slide rack into the wash tank containing Wash I at room temperature.
5. Remove a mixer-slide assembly from the Hybridization System and load it into the Mixer Disassembly Tool immersed in the shallow dish containing warm 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 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.

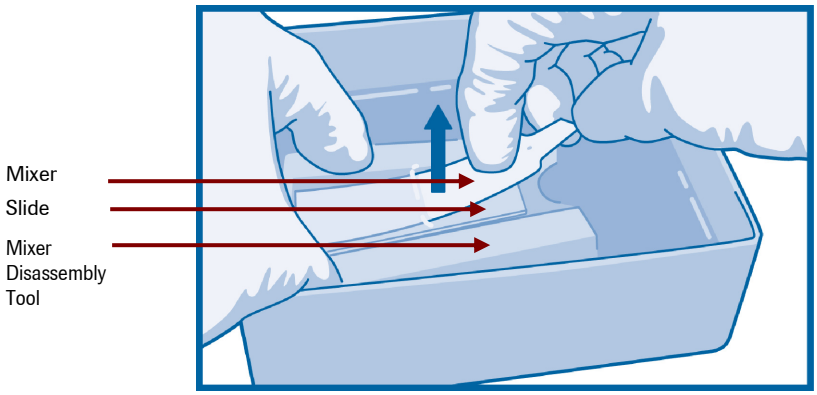


Figure 10: Using the Mixer Disassembly Tool to Remove a Slide from a Mixer

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 warm 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 Wash I. If washing one slide, transfer the slide into a slide container that contains 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.

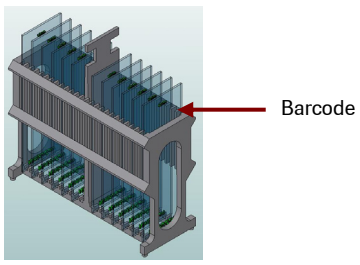


Figure 11: Insert Slides with the Barcode at the Top into the Slide Rack

If you are using a NimbleGen Microarray Dryer or other microarray dryer that dries multiple slides at a time, repeat steps 4 - 9 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 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. 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.

12. 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).

13. Remove the slide(s) from Wash III. Spin dry in a NimbleGen Microarray Dryer or other microarray dryer per the manufacturer's recommendation. For a NimbleGen Microarray Dryer, the recommended drying time is 2 minutes (120 seconds).

14. Remove the slide(s) from the NimbleGen Microarray Dryer or other microarray dryer. Blot dry the edges to remove any residual moisture.

    When not in use, store the dried slide in its original slide case in a dark desiccator.

15. Proceed immediately to the steps for scanning the array(s) in *Chapter 5*.

Notes

Chapter 5. Two-Color Array Scanning

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 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.

Keep arrays in a dark desiccator until you are ready to scan them. When handling slides, wear powder-free gloves and use care to touch only the slide's edges.

Step 1. Start Control Unit, Turn on Scanner & Load Slides

1. Start the control unit and log into your user account as msOperator or other account as set up by your system administrator.

   Account: msOperator
   Password: 1-msOperator

2. Turn on the scanner using the power switch on the left side.
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 aerosol compressed air for this purpose.
4. Insert slides into the Slide Magazine as described in Figure 12. Numbered slots provide spacing for inserting slides.

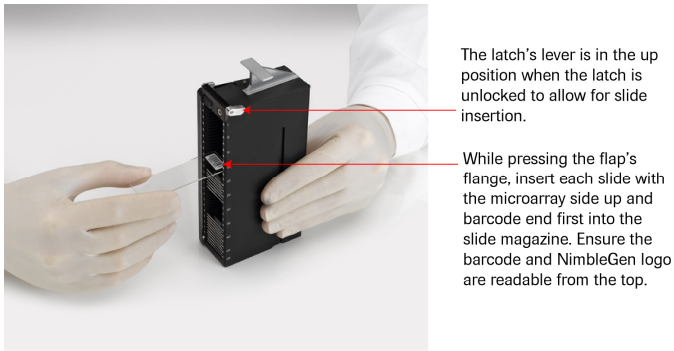


Figure 12: Inserting Slides into the Slide Magazine

5. Press the insert/eject magazine button on the scanner to open the stacker cover. Insert the slide magazine with loaded slides, aligning the slot on the slide magazine's side to join with the rail profile inside the scanner. The lowering of the slide magazine is interrupted by a mechanical hold point. Apply gentle pressure to complete insertion. Press the insert/eject magazine button to close the stacker cover (Figure 13). The initialization process starts, checking the slide magazine to determine which slots are occupied.

Figure 13: Inserting the Slide Magazine into the Scanner

Step 2. Start the Software & Turn on the Lasers

1. Double-click the NimbleGen MS200 icon to launch the MS 200 Data Collection Software. Make sure that the software has completely loaded before continuing.
2. Click the **Green Laser** and **Red Laser** buttons in the Laser Control (Figure 14) to switch on the lasers. Allow lasers to warm for 10 minutes.

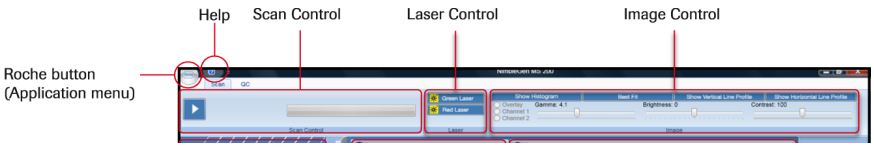


Figure 14: Top of Data Collection Workspace, showing Scan Control, Laser Control, and Image Control

3. Review the Magazine Control (Figure 15) in the Data Collection Software. Ensure that a green box appears in the *Slide Present* field for each slide loaded into the slide magazine.

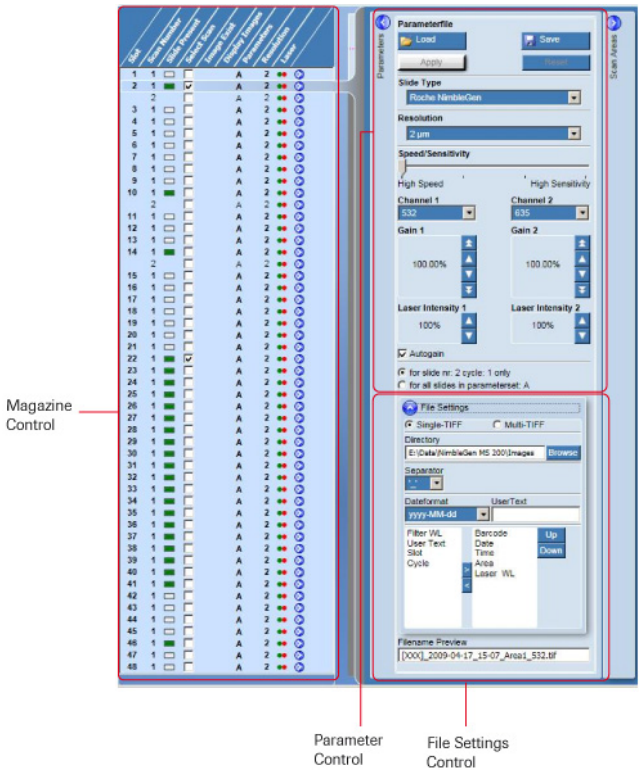


Figure 15: Magazine Control, Parameter Control, and File Setting Control

Step 3. Set Scan Parameters Using the Software

1. Use the Parameter Control (Figure 15) to set the parameters to use when scanning:
   a. To open the Parameter Control if not displayed, go to the row of a slide to scan in the Magazine Control and click its **Open Parameter Control** button ( ) to display the Parameter Control.
   b. Do not adjust the following default parameters:
      - Slide type
      - Channel 1 and Channel 2
      - Laser Intensity 1 and Laser Intensity 2
      - for slide nr: 2 cycle: 1 only
   c. Adjust the following default parameters if necessary:
      - Resolution
      - Speed/Sensitivity
      - Autogain
   d. To save any changes to the settings, click **Apply**.
   e. (Optional) Click **Save** to save settings to a parameters file, which allows the settings to be applied to other slides.
   f. (Optional) To process the slide multiple times using the same or different parameters, create up to 12 cycles.

To create a cycle, close the Parameter Control by clicking the **Close Parameter Control** button ( ). Go to the slide row in the Magazine Control, right-click, and select **Add Cycle**. Open the Parameter Control. Then specify and save parameters for the cycle as described above (1b - 1e).

2. Use the File Settings Control (Figure 15) to specify image file settings:

   a. To open the File Settings Control if not displayed, go to the row of a slide to scan in the Magazine Control and click its **Open Parameter Control** button ( ) (Figure 15). In the Parameter Control, click the **Open/Close File Settings** button ( ) to open the File Settings Control.

   b. Choose the *Single-TIFF* option button to generate one image file in Tagged Image File Format (TIFF, .tif) per channel.

   c. If necessary, change 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 open a dialog box to specify a location in the directory and click **OK** to confirm.

   d. Use the annotation list to add or change annotations to include in file names. Click an annotation in the left list box and then click the right arrow button ( ) to add to the right list box.

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

      <Barcode>_<User Text>_<Laser WL>.tif

      where "WL" means wavelength. To change the order of the annotations, select the annotation and click the **Up** or **Down** button. For "User Text," make sure to type the text in the *User Text* field.

      The *Filename Preview* text box of the Parameter Control displays the entire naming convention of the image file (.tif).

   e. Click **Apply** to confirm settings.

   f. (Optional) To save the file settings to a file, click **Save** in the Parameters Control (Figure 15).

3. Use the Area Definition Control (Figure 16; denoted as *Scan Areas* in the software interface) to set scan, barcode, and autogain areas.

   a. To open the Area Definition Control if not displayed, in the Parameter Control, click the **Open/Close Area Definition Control** button ( ) (shown on the right side of Figure 15 above the *Scan Areas* label in the software interface). Figure 16 shows the components of the Area Definition Control.

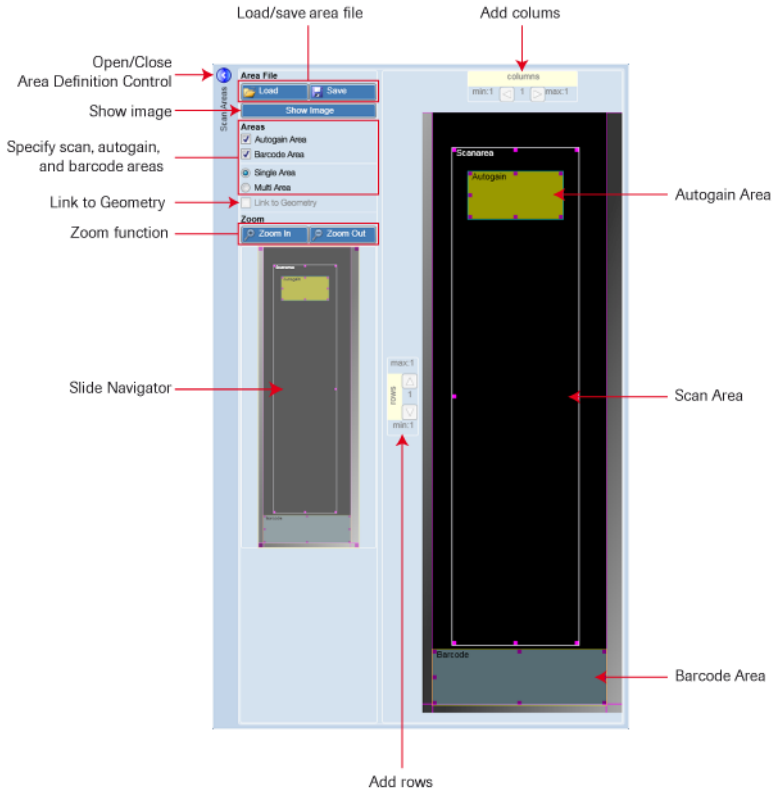


Figure 16: Components of the Area Definition Control

b. Ensure the following are selected:

- *Barcode area* checkbox
- *Autogain area* checkbox, if the Autogain checkbox was selected in the Parameter Control (Figure 15)
- *Single Area* option - this is the preferred option when scanning single and multiplex NimbleGen arrays. For multiplex arrays, you will use NimbleScan software's burst functionality to create individual image files for multiplex arrays.

c. Review and if necessary adjust the scan area:

- For NimbleGen 2.1M and 3x720K arrays, use the default selection for the scan area.
- (Optional) For NimbleGen 385K and 4x72K arrays, reduce the scan area, which will reduce the scan time. To define the scan area, download and use area files available at *www.nimblegen.com/scanner/* under *Download*. These files identify scan areas that are specific for NimbleGen 385K or 4x72K arrays. For instructions on how to use area files or how to manually adjust the scan area, refer to the *NimbleGen MS 200 Microarray Scanner Operator's Manual*.
- If desired, click **Save** to save settings to an area file for future use.

d. Review and if necessary adjust the autogain area.

To adjust the autogain area, position the mouse pointer inside the respective rectangle and click to enable the move cursor. Drag the rectangle to the desired array location and about 10 mm from the edge of the default scan area. Size the rectangle by dragging the side and corner handles (3 mm x 3 mm to 22 mm x 22 mm). Click **Apply** to confirm your settings.

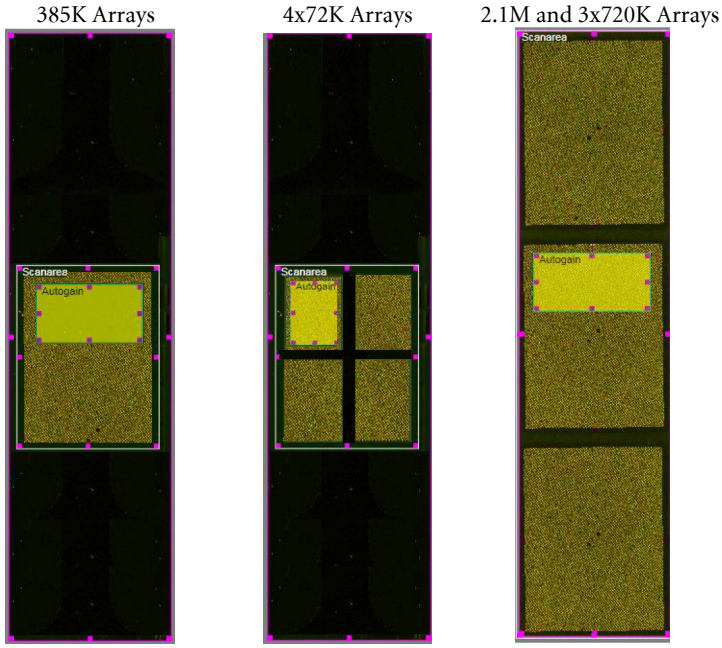


Figure 17: Examples of Autogain Areas for NimbleGen Arrays

4. Repeat the instructions above for each slide to process during the experiment.

   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. Scan the Slides

1. Click the **Start/Stop Scan** button ( ) in the Scan Control (Figure 18).
2. When prompted, specify the folder and file naming to save the session file.

   The scan process is then initiated. The Parameter Control and Area Definition Control close. The Image View, Slide View, and Spot View open (Figure 18), and the Scan Control shows the current task that is being performed above the progress bar.

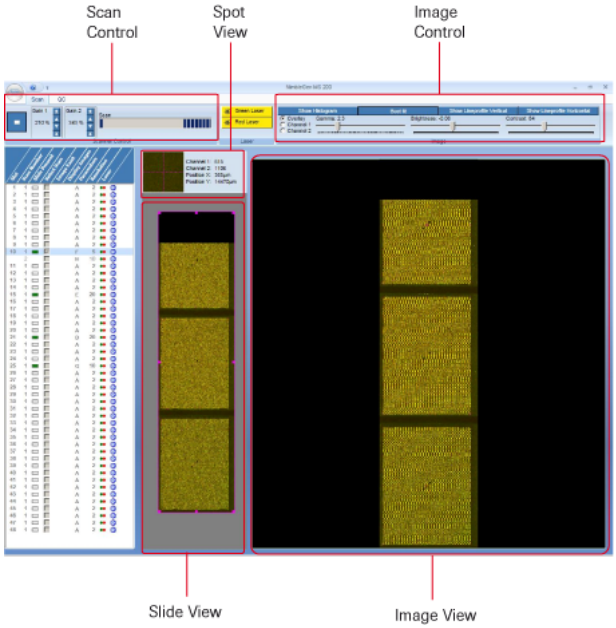
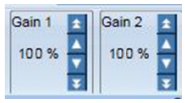


Figure 18: Example of a Run Time Window

3. (Optional) To manually adjust the PMT gain while scanning at 5 µm or higher resolution:

   a. Use the *Gain 1* and *Gain 2* spin boxes that appear in the Scan Control (Figure 18) to adjust the gain.



   b. Click **Show Histogram** in the Image Control (Figure 18) to view the histogram. Use the zoom feature (magenta box in the Slide View, Figure 20), to select where to view the histogram.

The best data are achieved when the red and green curves are superimposed or as close as possible to one another. If the red curve is above the green curve, lower the red curve (Channel 2). The curves should level out near 3 $\log_{10}$.

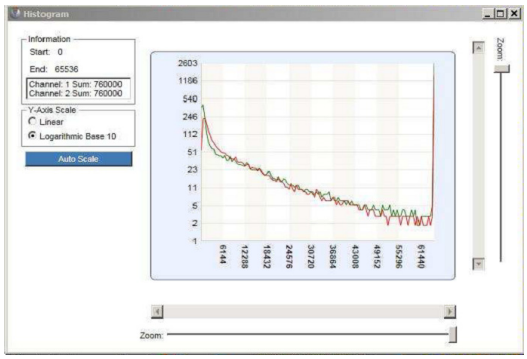


Figure 19: Histogram Window

c. Record the final gain settings to use to scan the slide again.

4. After the scanning experiment is completed, the light in the upper-left corner of the insert/eject magazine button on the scanner becomes green. The scanner unlocks its stacker cover, and you can remove the slide magazine.

Step 5. Review Scanned Images

1. Once the scan of a slide is completed, a blue **Displays Images** button ( ) appears in the *Display Images* field of the Magazine Control (Figure 15). Move the mouse pointer over the button to display the directory location and file name of the acquired image. Click the blue **Displays Images** button ( ) to display the image (both images) in the Image View.

2. To view a small region of the array and to select where to view the histogram (Figure 20), reduce the size of the magenta box that outlines the slide image in the Slide View so that the magenta box surrounds the area of interest. The area of interest will appear in the Image View. Click **Show/Hide Histogram** to display the histogram.

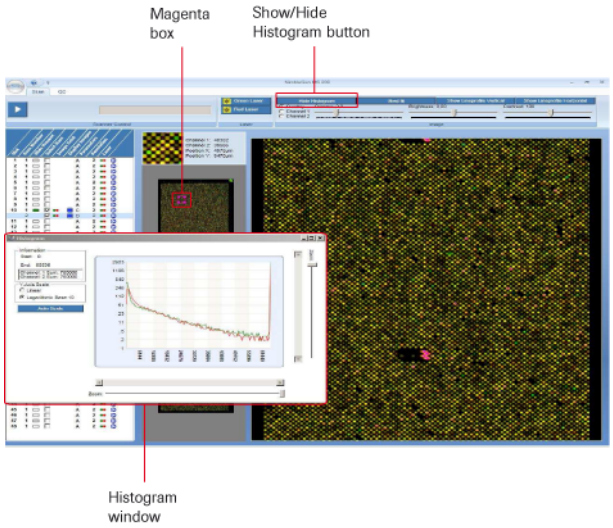


Figure 20: Example Window Showing Magenta Box in the Slide View, Show/Hide Histogram Button, and Histogram Window

3. Adjust contrast and brightness using the Image Control (Figure 21) to provide an improved view of features on the image. Adjusting these settings allows faint features to be more easily seen.

Figure 21: Image Control

Notes

Chapter 6. NimbleScan Data Analysis

Chapter 6 describes how to import a scanned image and extract the data using NimbleScan software. Refer to the *NimbleScan Software User's Guide v2.6* for computer system requirements and detailed information on using the software.

Step 1. Burst Multiplex Image (4x72K & 3x720K Arrays Only)

If your files contain the scanned images of 4x72K or 3x720K arrays, follow the steps below to burst (separate) each slide image into separate array images. Otherwise, proceed to "Step 2. Import Image."

1. Select **File** -> **Burst Multiplex Image**. The Burst Multiplex Image dialog box appears.
2. To choose the .tif files to burst, click **Add images**.
3. Navigate to the directory containing your array image file (.tif) and select the file.
4. Click **Add to batch**. Add both 532 and 635 images for each slide. Multiple slide images can be burst at once.
5. Browse to select the correct multiplex description file (.ncd) on the Design Information disk provided with the 4x72K or 3x720K array for the *Multiplex description file* field.
6. Browse to select the desired output file destination in the *Output burst images to* field.
7. Click **Burst**. NimbleScan software creates 4 or 3 image files. The array designation (A01 - A04 for 4x72K and A01 - A03 for 3x720K arrays) for each bursted image is specified in the original image's file name. For example:
   - <NNNNNA01>_<XXXXX>_<WWW>.tif…<NNNNNA04>_<XXXXX_WWW>.tif
   - <NNNNNA01>_<XXXXX>_<WWW>.tif…<NNNNNA03>_<XXXXX_WWW>.tif

   where **NNNNN** is the slide's barcode number, **XXXXX** is optional user-defined text, and **WWW** is the wavelength (532 or 635).
8. Repeat steps 1 - 7 for each multiplex image.

Alternatively, you can burst all images designed with the same layout file in a batch mode by selecting all files to burst in step 2 above.

Step 2. Import Image

1. Select **File** -> **Open**. The Open an Alignment dialog box appears.

Open and grid the 532 and 635 images separately.

2. Navigate to the directory containing your array images and select one image.
3. Select the design file (.ndf) in the Design Information disk provided with your array. This file describes the placement of the probes on the array.
4. Click **Open**. For 2.1M arrays, NimbleScan software will split the image into 3 subarrays and display each subarray under a separate tab.

Step 3. Extract Image

1. Select the auto brightness/contrast adjust function.

2. Select the auto align tool to overlay the grid on the array. Alternatively, select **Analysis** -> **Auto Align**. For 2.1M arrays, the auto align feature will overlay the grid on all 3 subarrays at once.

If the software indicates auto alignment was not successful, perform a manual alignment as described in the *NimbleScan Software User's Guide*.

3. Zoom into the top corner of the array with the zoom tool. Check to make sure all fiducial controls align correctly with the grid. For 2.1M arrays, check grid placement for the images on the *A01*, *A02,* and *A03* tabs and adjust if necessary.

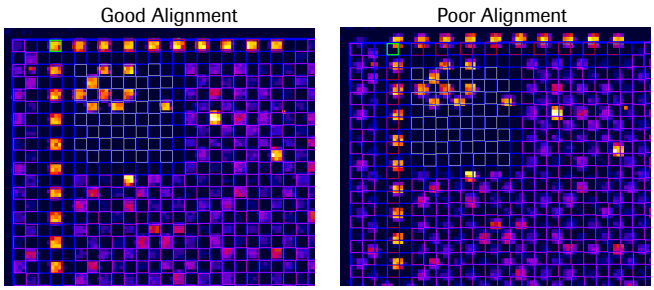


Figure 22: Examples Showing Good and Poor Grid Alignments

4. If necessary, adjust the grid by clicking on the green highlighted square located in the corner. Move this square so that the corner fiducial controls line up correctly with the grid. Move to each corner using the jump to location buttons. For 385K, 2.1M, and 3x720K arrays, also check the center fiducial controls.

5. Select **File** -> **Save** to save the gridded image.

6. If you are analyzing data from a 4x72K or 3x720K array, proceed to "Step 4. Confirm Experimental Integrity (4x72K & 3x720K Arrays Only)." If you are analyzing data from a 385K or 2.1M array, proceed directly to "Step 5. Generate an Experimental Metrics Report."

Step 4. Confirm Experimental Integrity (4x72K & 3x720K Arrays Only)

A unique STC should have been added to each experimental (IP) and control (input) sample pair prior to loading onto 4x72K or 3x720K 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 23). Roche NimbleGen recommends performing a sample tracking analysis (refer to page 42) and visually checking the STC features along the perimeter (refer to page 44) to confirm that the correct sample has been added to each array.

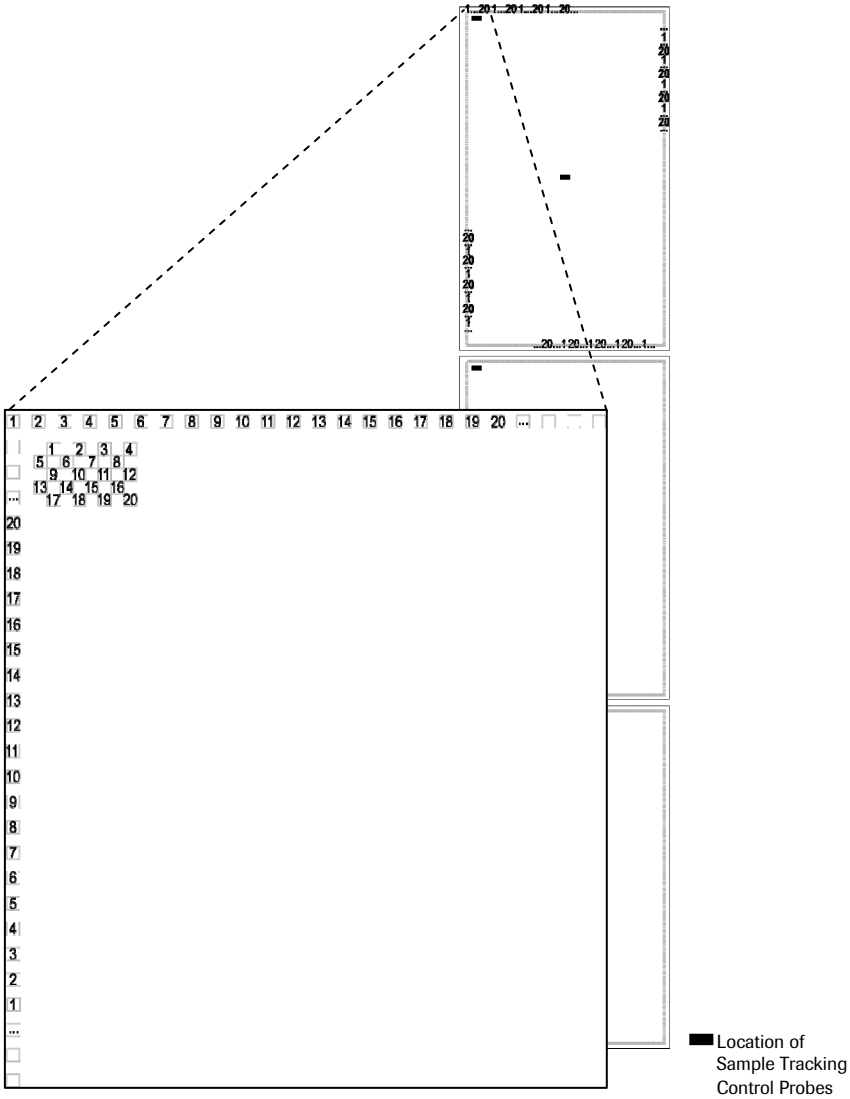


Figure 23: Location and Numbering of Sample Tracking Control Probes on a 3x720K Array

Performing a Sample Tracking Analysis

When you run a Sample Tracking analysis, NimbleScan software generates a Sample Tracking report based on signal intensities of features in the two 4 x 5 blocks with Sample Tracking Control probes (Figure 23). Using this report and sample records from your experiment, you can confirm the intended sample was hybridized to the intended array.

1. Select **Analysis** -> **Sample Tracking**.
2. Click **Add files**.
3. Select all gridded 532 nm images for the experiment (for example, select 4 images for 4x72K arrays).
4. Click **Add to List**.
5. Click **Browse** to select the destination of the output file. To view the output file in Microsoft Excel, save as an .xls file.
6. Click **Run** to start the analysis.
7. Open the Sample Tracking report in spreadsheet software, such as Microsoft Excel. An example report is shown in Figure 24.
    - Confirm that the Image ID, Design ID, and Design Name are accurate.
    - Enter the Sample Name and Input STC in the appropriate spreadsheet cells. The Input STC is the unique STC number added to each sample before hybridization.
    - For each array, review the signal intensity and the Absent or Present call for each of the up to 20 STCs that Roche NimbleGen may provide. The STC identified as Present should be the same as the Input STC you entered for the sample.
    - Ensure that a Present call is reported for only the unique STC added to the sample. If more than one Present call is reported, the integrity of your data could be compromised due to cross-contamination that occurred during sample preparation, loading, or hybridization. The extent of cross-contamination that can be tolerated depends on your samples, experimental setup, and experimental goals. Roche NimbleGen recommends repeating experiments that show cross-contamination.
    - Select **File** -> **Save** to save the changes to the file.

Image ID	109037A01_510_532	109037A02_510_532	109037A03_510_532	109037A04_510_532
Design ID	5010	5010	5010	5010
Design Name	HG18_60mer_expr	HG18_60mer_expr	HG18_60mer_expr	HG18_60mer_expr
Sample Name				
Array Number	A01	A02	A03	A04
Input STC				
STC 1 Intensity	39528	210	210	218
STC 2 Intensity	215	228	253	207
STC 3 Intensity	182	48920	232	206
STC 4 Intensity	191	214	221	250
STC 5 Intensity	195	248	40937	244
STC 6 Intensity	183	242	195	216
STC 7 Intensity	200	252	212	36140
STC 8 Intensity	202	198	212	187
STC 9 Intensity	186	213	208	199
STC 10 Intensity	223	240	203	214
STC 11 Intensity	226	215	216	237
STC 12 Intensity	171	240	185	207
STC 13 Intensity	199	225	206	209
STC 14 Intensity	176	217	264	223
STC 15 Intensity	207	240	233	207
STC 16 Intensity	168	242	206	195
STC 17 Intensity	239	252	268	258
STC 18 Intensity	225	227	212	207
STC 19 Intensity	211	226	219	221
STC 20 Intensity	259	239	228	221
STC 1 Call	Present	Absent	Absent	Absent
STC 2 Call	Absent	Absent	Absent	Absent
STC 3 Call	Absent	Present	Absent	Absent
STC 4 Call	Absent	Absent	Absent	Absent
STC 5 Call	Absent	Absent	Present	Absent
STC 6 Call	Absent	Absent	Absent	Absent
STC 7 Call	Absent	Absent	Absent	Present
STC 8 Call	Absent	Absent	Absent	Absent
STC 9 Call	Absent	Absent	Absent	Absent
STC 10 Call	Absent	Absent	Absent	Absent
STC 11 Call	Absent	Absent	Absent	Absent
STC 12 Call	Absent	Absent	Absent	Absent
STC 13 Call	Absent	Absent	Absent	Absent
STC 14 Call	Absent	Absent	Absent	Absent
STC 15 Call	Absent	Absent	Absent	Absent
STC 16 Call	Absent	Absent	Absent	Absent
STC 17 Call	Absent	Absent	Absent	Absent
STC 18 Call	Absent	Absent	Absent	Absent
STC 19 Call	Absent	Absent	Absent	Absent
STC 20 Call	Absent	Absent	Absent	Absent

Figure 24: Example of a Sample Tracking Report

Visually Checking STC Features

1. If necessary, zoom into the upper left corner of the array with the zoom tool.

2. Locate the repeating set of 20 features along the perimeter of the array. Figure 25 shows an example of an array hybridized with a sample containing STC 5.

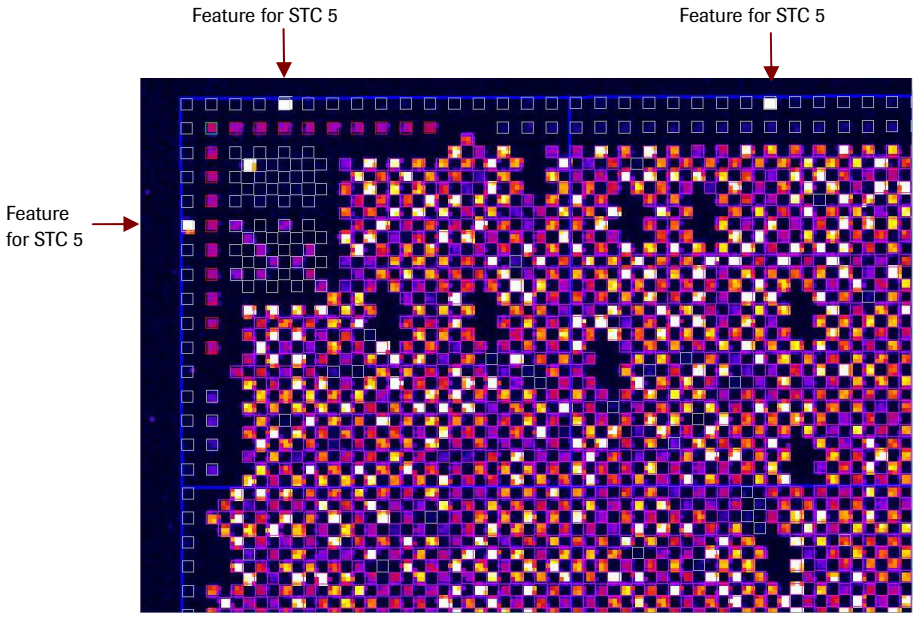


Figure 25: Example of an Array Hybridized with Sample Containing STC 5

3. 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 repeating experiments that show cross-contamination.

Step 5. Generate an 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* provided with NimbleScan software.

1. Select **Analysis -> Generate Experimental Metrics Report**.

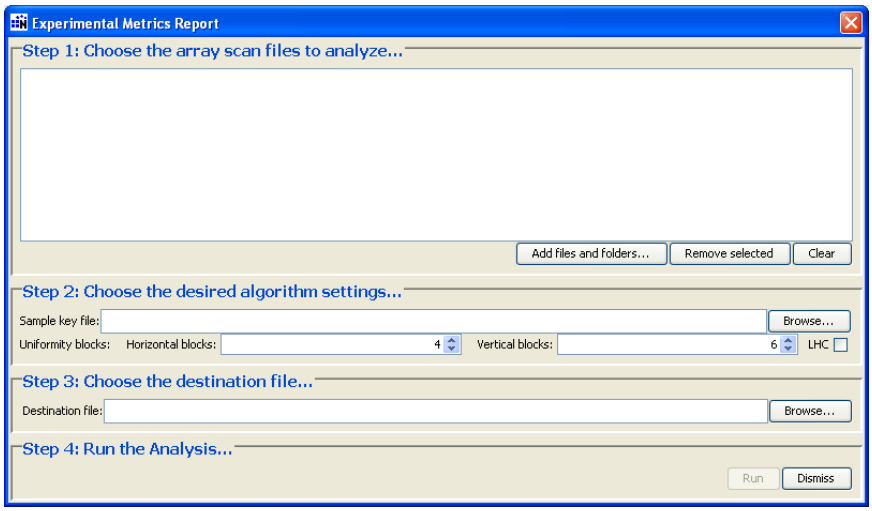


Figure 26: Experimental Metrics Report Dialog Box

2. In *Step 1: Choose the array scan files to analyze…*:
   - List box. Identifies the files to analyze.
   - Click **Add files and folders** to select all gridded images (.tif) for the experiment (for example, select 4 images for 4x72K arrays). When the Open dialog box opens, navigate to the location of the files. You can select multiple files by pressing the Ctrl key and clicking each file name.
   - If you choose the wrong file, click the file name in the list box, and click **Remove selected**.
   - To remove all selected files, click **Clear**.
3. In *Step 2: Choose the desired algorithm settings…*:
   - (Optional) Click **Browse** to select the sample key file.
   - (Optional) Adjust the settings in the *Uniformity blocks* fields. For 2.1M arrays, Roche NimbleGen recommends the following settings: 4 for horizontal and 18 for vertical. For all other array formats (385K, 4x72K, or 3x720K arrays), we recommend the default settings (4 for horizontal and 6 for vertical).
4. In *Step 3: Choose the destination file…*, type the path to the folder in the *Destination file* field or click **Browse** to open a dialog box for navigating to the destination folder.
5. In *Step 4: Run the Analysis…*, click **Run** to start the analysis.
6. Open the Experimental Metrics report in spreadsheet software, such as Microsoft Excel. An example report is shown in Figure 27.

IMAGE_NAME	INTERQUARTILE_RANGE	DENSITY	RATIO_RANGE	SIGNAL_RANGE	UNIFORMITY_MEAN	UNIFORMITY_CV	NUM_EMPTY	MEAN_EMPTY	NUM_EXPERIMENTAL	MEAN_EXPERIMENTAL	NUM_RAN_DOM	MEAN_RAN_DOM	LHC_2X	LHC_3X	LHC_4X	LHC_SLOPE	LHC_DEL
365182A02_532.tif		1.467	0.24	0.233	2222.206	0.07	741805	949.29	716686	2222.446	3000	689.821	n/a	n/a	n/a	n/a	n/a
365182A02_635.tif		1.932	0.24	0.415	2361.751	0.088	741805	1149.077	716686	2361.806	3000	651.998	n/a	n/a	n/a	n/a	n/a
365182A03_532.tif		1.431	0.343	0.212	2288.962	0.064	741805	905.255	716686	2289.164	3000	685.668	n/a	n/a	n/a	n/a	n/a
365182A03_635.tif		1.992	0.343	0.517	2436.78	0.125	741805	1166.569	716686	2436.928	3000	700.622	n/a	n/a	n/a	n/a	n/a
367935A01_532.tif		1.612	0.247	0.268	2113.519	0.062	741805	820.131	716686	2113.958	3000	596.564	n/a	n/a	n/a	n/a	n/a
367935A01_635.tif		1.965	0.247	0.394	2200.912	0.08	741805	1050.552	716686	2201.133	3000	615.092	n/a	n/a	n/a	n/a	n/a
367935A02_532.tif		1.587	0.262	0.219	2375.713	0.06	741805	978.455	716686	2376.138	3000	664.324	n/a	n/a	n/a	n/a	n/a
367935A02_635.tif		2.032	0.262	0.443	2429.347	0.094	741805	1186.245	716686	2429.701	3000	686.826	n/a	n/a	n/a	n/a	n/a
367935A03_532.tif		1.469	0.265	0.301	2379.192	0.088	741805	953.592	716686	2379.542	3000	727.569	n/a	n/a	n/a	n/a	n/a
367935A03_635.tif		1.892	0.265	0.562	2349.683	0.132	741805	1174.794	716686	2349.92	3000	694.712	n/a	n/a	n/a	n/a	n/a

Figure 27: Example of an Experimental Metrics Report

Following is a description of the contents of the Experimental Metrics report:

- *IMAGE_NAME*. The name of the analyzed image file.
- *INTERQUARTILE_DENSITY*. The interquartile range (IQR) of the raw signal intensities.
- *RATIO_RANGE*. The ratio range for two-color microarray applications. Ratio range is calculated by dividing the array into a grid of uniformity blocks (4 x 18 on 2.1M arrays and 4 x 6 on 385K, 4x72K, or 3x720K arrays), calculating the average $\log_2$ ratio of each block and calculating the range from the block averages.

  This metric applies only to two-color applications.

- *SIGNAL_RANGE*. The signal range represents the signal range on a per channel basis for both one- and two-color microarray applications. Similar to ratio range, the signal range is calculated based on the range of signal means from the average signal per block.
- *UNIFORMITY_MEAN*. The mean signal intensity of all the probes in each uniformity block.
- *UNIFORMITY_CV*. The coefficient of variation of the block uniformity means.
- *NUM_EMPTY*. The number of empty features present on the array.
- *MEAN_EMPTY*. The mean signal intensity of empty features present on the array.
- *NUM_EXPERIMENTAL*. The number of experimental features present on the array.
- *MEAN_EXPERIMENTAL*. The mean signal intensity of the experimental features present on the array.
- *NUM_RANDOM*. The number of random control features present on the array.
- *MEAN_RANDOM*. The mean signal intensity of the random control features present on the array. These probes generally have the same length and GC characteristics as the experimental probes on the array, and can be used to estimate the amount of non-specific binding in the hybridization.

Disregard the LHC and SLOPE metrics provided on the report. LHC controls are not used in DNA methylation analysis.

Step 6. (Optional) Create Pair Reports

In version 2.4 or earlier of NimbleScan software, pair reports (.pair files) were used as input for data analysis. In later versions of the software, data analysis ("Step 7. Create Scaled Log_2 Ratio Data") can use either .pair or gridded .tif files (generated earlier in "Step 3. Extract Image") as input.

Pair reports (.pair), the raw data format for NimbleGen DNA Methylation experiments, contain the signal intensities for each probe on the array. Create two Pair reports for each array, one for the Cy3 image and one for the corresponding Cy5 image. Images from arrays with the same design can be analyzed in batch mode.

1. Select **Analysis** -> **Reports** -> **Pair**.

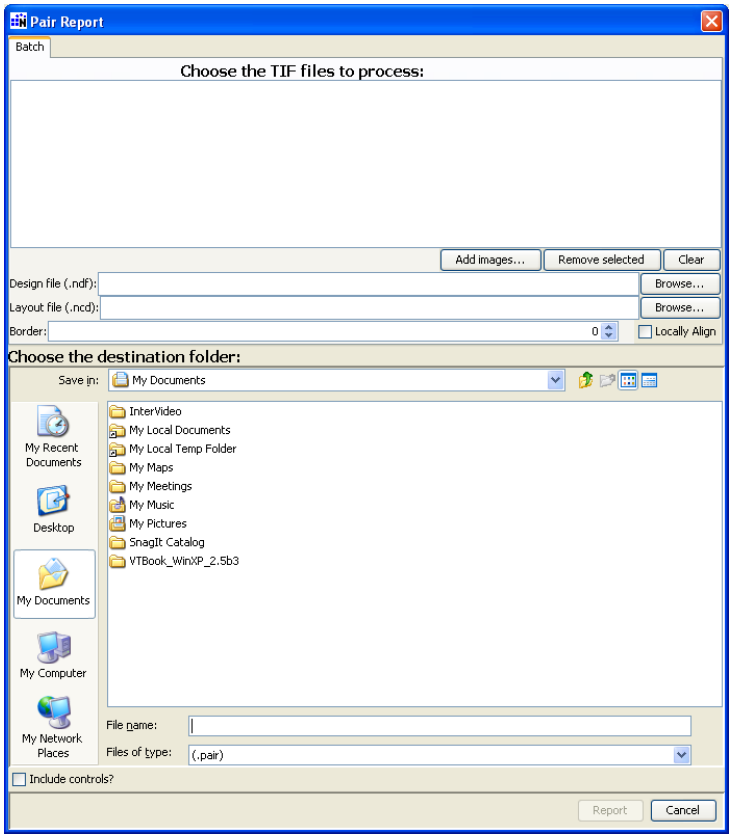


Figure 28: Pair Report Dialog Box

2. Choose the image files (.tif) to process:
   - Click **Add images** to select image files (.tif). When the Open dialog box opens, you can navigate to the location of the image files. Select multiple files by pressing the Ctrl key and clicking each file name.
   - If you choose the wrong file, click the file name in the list box, and click **Remove selected**.
   - To remove all selected files, click **Clear**.
3. Browse to and select the appropriate design file name in the *Design file (.ndf)* field and for 2.1M arrays, the layout file name in the *Layout file (.ncd)* field. The .ndf and .ncd files are included on the CD/DVD shipped with your arrays.
4. (Optional) Fine-tune feature quantification:

   Locally Align. When selected, the software shifts a quantification square (without overlapping the adjacent features) until it finds the maximum intensity for the feature.

   Local alignment is not recommended for 2.1M or 3x720K arrays.
5. Choose where to save the reports in the *Save in* field.
6. Choose the *Include controls?* option if you want the software to report the raw data for the fiducial controls and Sample Tracking Controls.
7. Click **Report** to generate the report(s).

Step 7. Create Scaled Log_2 Ratio Data

1. Select **Analysis -> Methylation -> Compute Ratio Files.**

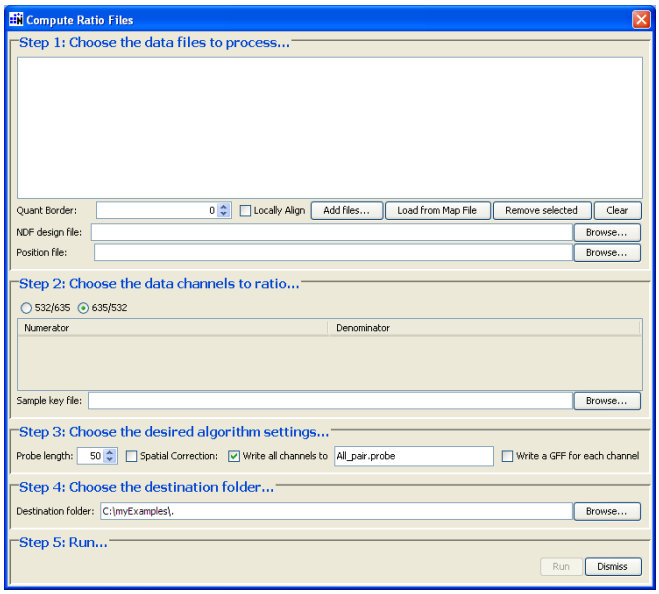


Figure 29: **Compute Ratio Files Dialog Box**

2. In *Step 1: Choose the data files to process...:*

   - List box. Identifies the files to process.
   - Click **Add files** to select either the .pair file(s) generated in "Step 6. (Optional) Create Pair Reports" or the gridded .tif files generated in "Step 3. Extract Image" to process. When the Open dialog box opens, navigate to the location of the files. You can select multiple files by pressing the Ctrl key and clicking each file name.
   - If you choose the wrong file, click the file name in the list box, and click **Remove selected**.
   - To remove all selected files, click **Clear**.

     All .pair files selected must be from arrays that contain the same design.

   - *Position file*. Select the positions file (.pos) that corresponds to the raw data .pair files specified in Step 1 of the dialog box. The positions file maps each probe on the array to its genomic location. Each microarray design has a single corresponding positions file on the CD/DVD shipped with your microarrays.

3. In *Step 2: Choose the data channels to ratio...:*

   - *532/635* or *635/532*. Use the default 635/532 setting.
   - (Optional) *Sample key file*. Select the sample key file.

4. In *Step 3: Choose the desired algorithm settings...:*

   - *Probe length*. If needed, change the probe length (normally 50 bp). Note that if the positions file, described earlier, identifies the probe length, the software automatically uses that value.

The probe length is reflected in the width of probes in the GFF files generated by the analysis.

- *Write all channels to All_pairs.probe.* Choose whether to create an All Pairs Probe (.probe) file that provides sequence identifier, probe identifier, and signal intensity for all files in the analysis.
- *Write a GFF for each channel.* Choose whether to create a separate GFF (General Feature Format, .gff) file that includes signal intensity information for each channel.

5. In *Step 4: Choose the destination folder...*, type the path to the folder in the *Destination folder* field or click **Browse** to open a dialog box for navigating to the destination folder.
6. In *Step 5: Run...*, click **Run**. It will take some time to complete the analysis. Expect processing for each 2.1M array to take at least 5 - 20 minutes to complete based on computer speed.
7. Go to the destination folder that contains the analyzed data and review the files.

File Format	Description
GFF file (.gff)[1]	(General Feature Format) A GFF file is an exchange format for genomic-based data. A DNA methylation GFF file contains scaled $\log_2$ ratio data, if available.
All Pairs Probe file (.probe)[2]	Provides sequence identifier, probe identifier, and signal intensity for multiple data channels in the same row.

1 Open with SignalMap software to view data graphically or with a text editor to view data in a table format.

2 View using a text editor, such as Microsoft WordPad.

Step 8. Find Peaks

1. Select **Analysis** -> **Methylation** -> **Find Peaks**.

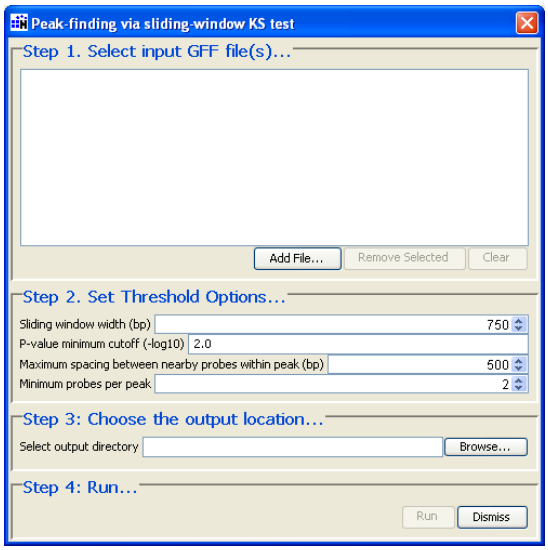


Figure 30: Peak-finding via sliding window KS test Dialog Box

2. In *Step 1: Select input GFF file(s)…*:

- Click **Add files** to select the ratio data files (.gff) generated in the previous section, "Step 7. Create Scaled Log_2 Ratio Data."
  When the Open dialog box opens, navigate to the location of the files. You can select multiple files by pressing the Ctrl key and clicking each file name.
- If you choose the wrong file, click the file name in the list box, and click **Remove selected**.
- To remove all selected files, click **Clear**.

3. In *Step 2: Set Threshold Options…*, leave the default settings for the following parameters:
   - *Sliding window width (bp)*. Adjusts the width of the sliding window surrounding each probe. Increasing this value tests more points around each probe against the rest of the array. This in turn gives additional statistical power for resolving positive enrichment regions at the expense of lower resolution. The default setting is 750 bp.
   - *P-value minimum cutoff (-log10)*. Probes scoring above this cutoff (default set at 2) comprise peaks. Higher $-\log_{10}$ values increase stringency and result in fewer peaks being identified.
   - *Maximum spacing between nearby probes within peak (bp)*. Nearby probes equal to or less than the specified spacing are consolidated into a single peak. This parameter specifies the maximum distance over which high-scoring probes are joined to form peaks. The default setting is 500 bp. Increasing the distance between nearby probes (e.g. 1,000 bp) within peak may merge peaks that may otherwise be identified as separate peaks.
   - *Minimum probes per peak*. Specifies the minimum number (default set at 2) of probes that must be above the cutoff before the region is identified as a peak. Increasing the minimum probes per peak (e.g. 4 probes) increases the stringency of peak calling because a peak requires more probes to comprise that peak.
4. In *Step 3: Choose the output location…*, type the path to the folder in the *Select output directory* field or click **Browse** to open a dialog box for navigating to the destination folder.
5. In *Step 4: Run…*, click **Run**. It will take some time for the analysis to complete. Expect a processing time of at least 20 minutes for each 2.1M array when using the default analysis settings.

Step 9. Map Peaks

1. Select **Analysis** -> **Methylation** -> **Map Peaks**.

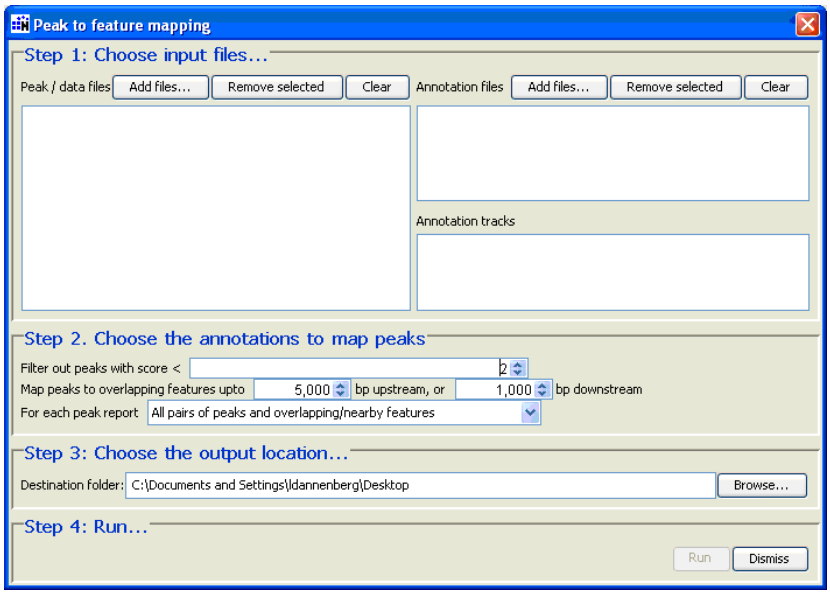


Figure 31: Peak to feature mapping Dialog Box

2. In *Step 1: Choose input files…*:

   a. Click **Add files** above the *Peak / data files* list box and choose the peak data file(s) generated in the previous section, "Step 8. Find Peaks."

   b. Click **Add files** above the *Annotation files* list box and choose the annotation file(s) with the feature information to which to map peaks. The software automatically starts scanning the file(s) for annotation tracks and lists them in the *Annotation tracks* list box.

   c. In the *Annotation tracks* list box, select "transcription start site" to map peaks to the +1 site of each gene. You may also select other annotation tracks to map peaks to that feature (e.g. CpG islands).

3. In *Step 2: Choose the annotations to map peaks,* leave the default settings for the following parameters:

   - *Filter out peaks with score <*. This parameter eliminates peaks of low confidence from the summary report.
   - *Map peaks to overlapping features up to*. This parameter sets the limit on how far upstream or downstream from a given feature the peak must be located in order to consider it a mapped peak. Be certain to adjust the upstream and downstream values based on the array design being used.
   - *For each peak report.* The "All pairs of peaks" selection generates a report listing every combination of peak and annotation record that map together. The "Nearest feature (if any) to each peak" selection generates a report listing only the nearest feature to a peak. The "Nearest peak (if any) to each feature" selection generates a report listing only the nearest peak to a feature.

4. In *Step 3: Choose the output location…*, type the path to the folder in the *Destination folder* field or click **Browse** to open a dialog box for navigating to the destination folder.

5. In *Step 4: Run…*, click **Run**.

6. (Optional) Repeat steps 1 - 5 with these changes:
   a. Select "Nearest peak (if any) to each feature" in the *For each peak report* field. This report generates a report listing only the nearest peak to a feature.
   b. Choose a different output location than selected in the previous analysis to prevent overwriting of summary reports.

Step 10. Learn More about Your Data

Signal Intensity (Raw) Data

Signal intensity data is extracted from the scanned images of each array using NimbleScan software. Signal intensities for each probe are saved in pair files (.txt), the raw data format for DNA methylation experiments. View pair files using a text editor. (Refer to "Step 6. Create Pair Reports" on page 47 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. View $\log_2$ ratio data files (.gff) using SignalMap software. (Refer to "Step 7. Create Scaled Log_2 Ratio Data" on page 49 for information on how to create $\log_2$ ratio data files.)

P-Value Data

From the scaled $\log_2$ ratio data, a fixed-length window (default = 750 bp) is placed around each consecutive probe and the one-sided Kolmogorov-Smirnov (KS) test is applied to determine whether the probes are drawn from a significantly more positive distribution of intensity log ratios than those in the rest of the array. The resulting score for each probe is the $-\log_{10}$ p-value from the windowed KS test around that probe. (Refer to "Step 8. Find Peaks" on page 50 for information on how to create p-value data files.)

Peak Data

Using NimbleScan software, peak data files (.gff) are generated from the p-value data files. NimbleScan software detects peaks by searching for the user-specified number of probes (default = 2) above a user-specified p-value minimum cutoff ($-\log_{10}$, default = 2) and merges peaks within a user-specified distance (default = 500 bp) of each other. View peak data files graphically or in table format using SignalMap software or a spreadsheet software, respectively. (Refer to "Step 8. Find Peaks" on page 50 for information on how to create peak data files.)

Viewing Peak Data Graphically

In SignalMap software, position the mouse pointer over each peak to display additional information (see table below).

Field	Description
Score	The peak score, which is the average $-\log_{10}$ p-values from probes within that peak.
Pos	Genomic coordinates of the peak.

Viewing Peak Data in a Table Format

You can also open peak data files (.gff) in a spreadsheet program, such as Microsoft Excel, to view data in a table format. See the table above for a detailed description of peak data.

Summary Reports

For each annotated gene, NimbleScan software searches for peaks that appear in a specified promoter region around the transcription start site (TSS). The region searched is design-specific. For most mammalian designs, the search region spans from 5 kb upstream to 1 kb downstream of the TSS. The region spanned can be adjusted based on the array design. (Refer to "Step 9. Map Peaks" on page 51 for information on how to create summary reports.)

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

- ***ArrayID*_ratio_peaks_mapToFeatures_All_Peaks.xls:** Lists all peaks and maps them to promoter regions. Each row in the report lists a peak-transcript pair. For each transcript, if more than one peak lies within the promoter region, there will be multiple rows for that transcript.
- ***ArrayID*_ratio_peaks_mapToFeatures_Nearest_Peak.xls:** Lists all peaks and maps them to promoter regions. Each row in the report lists a peak-transcript pair. For each transcript, if more than one peak lies within the promoter region, only the peak nearest to the TSS is reported.

To effectively analyze peak data, you should sort the data in summary reports according to peak score, gene name, chromosome, distance to TSS, etc. To sort data in Microsoft Excel, highlight row 1 and select **Data -> Filter -> Auto Filter**. You can then sort individual columns by ascending/descending values, top 10 values, or individual values.

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

Field	Description
PEAK_ID	An ID for each peak.
CHROMOSOME	Chromosome associated with the peak.
PEAK_START	First base of the peak on the chromosome.
PEAK_END	Last base of the peak on the chromosome.
PEAK_SCORE	The peak score, which is the average $-\log_{10}$ p-values from probes within that peak.
FEATURE_TRACK	The annotation track against which peaks were mapped; it is the transcription start site for summary 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.*
FEATURE_TO_PEAK_DISTANCE	Center-to-center distance of peak to feature.
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.
PEAK_ATTR	Attribute field from the peak GFF file.

Control Regions on NimbleGen Human and Mouse DNA Methylation 2.1M and 3x720K Arrays

For NimbleGen Human and Mouse DNA Methylation 2.1M and 3x720K arrays, probes for three types of control regions are included on each slide. These control regions are displayed as independent tracks in the .gff files containing the scaled $\log_2$ ratio, p-value, and peak data. Each probe for a control region is represented three times across the array. An example of positive control and experimental results are shown in Figure 32.

- **Positive control regions:** Represent regions of known DNA methylation in normal somatic tissues, such as H19, GNAS, TSH2B (HIST1H2BA), and XIST, as well as regions derived from retroviral LTRs.
- **Negative control regions:** Derived from multiple histone genes that exhibit minimal or no DNA methylation in normal somatic tissues.
- **NonCG control regions:** Correspond to ≥ 1 kb genomic regions with no CpG dinucleotides and are included to illustrate the background signal level derived from completely unmethylated genomic regions.

These control regions assist in analyzing performance of a DNA methylation experiment. In most normal tissues, the positive control regions should identify methylated DNA fragments within the experimental sample, and the negative control regions should show minimal DNA methylation. The nonCG control regions contain no CpG sites. Therefore, these probes should not identify methylated DNA fragments in the experimental samples.

Results from positive and negative control regions could vary depending on the tissue studied and/or cellular treatment. For example, the TSH2B positive control region is methylated in most tissues tested except for testis or lung cancer research samples. Similarly, tissues exposed to demethylating reagents such as 5-aza-2-deoxycytidine will result in loss of methylation in expected regions. Thus, the control regions are not to be used as an indisputable quality measurement for any given array or MeDIP experiment but as a point of reference from known DNA methylation patterns found in normal healthy tissues.

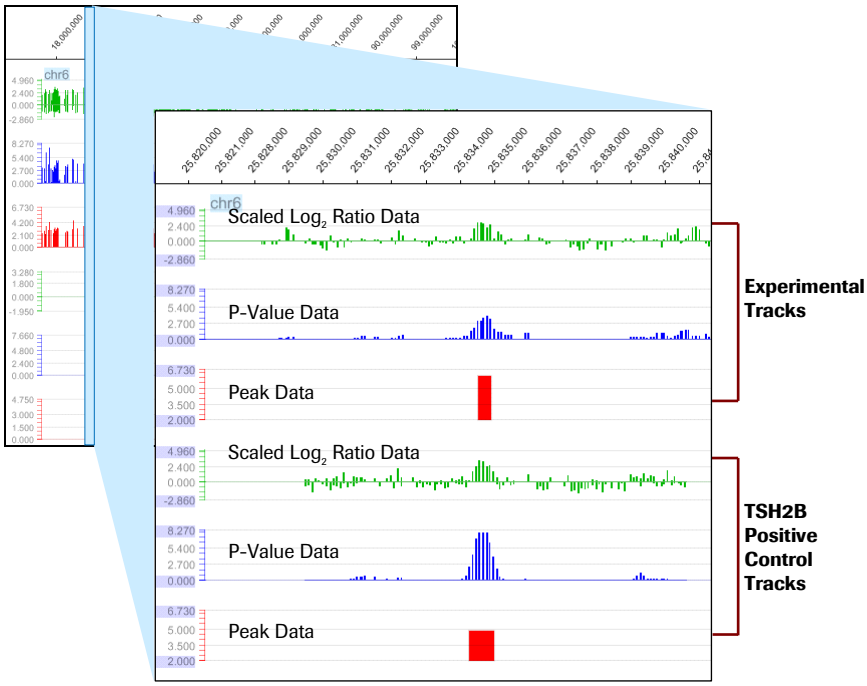


Figure 32: Example of Experimental and Positive Control Tracks from Human Chromosome 6. The three experimental tracks (scaled $\log_2$ ratio, p-value, and peak data) represent the results obtained from a typical DNA methylation experiment. The three positive control tracks (scaled $\log_2$ ratio, p-value, and peak data) represent the results for the set of probes targeted to the TSH2B positive control region.

Be aware of the following: 1) Although positive, negative, and nonCG control probes may cover the same genomic region as the experimental probes, the control and experimental probe sequences may not match exactly. In the case of the above example, the probe sequences are not the same. 2) If negative and nonCG control probes covered the region that is illustrated, their data tracks would also be displayed. For simplicity, the negative and nonCG tracks are not shown in this example.

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 summary reports being generated. In addition, the gene description file (.ngd) is available only for certain designs, since these files were replaced by annotation files (.gff) in newer designs. Also, if a positions file (.pos) is not available (because genomic coordinates were not provided for a custom design), no scaled $\log_2$ ratio files (.gff), peak data files (.gff), or summary reports (.xls) are generated.

Notes

Chapter 7. Troubleshooting

This chapter 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.

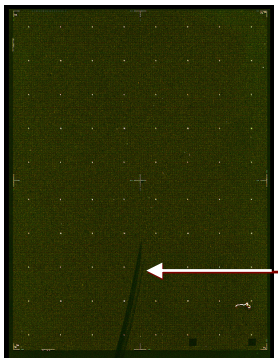
Labeling

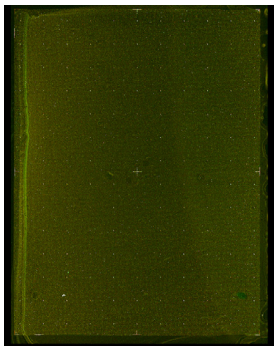
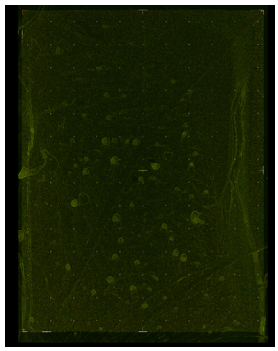
Problem	Possible Cause(s)	Recommended Corrective Action
Labeling yield is less than 8 µg per reaction (or less than 24 µg total for 2.1M arrays).	DNA sample is contaminated or degraded.	Check absorption ratios and gel image. If necessary, clean up samples or repeat the ChIP protocol. Repeat labeling.
	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 -20°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.
Pellets are not solid.	Incorrect ratio of water, isopropanol, and salt.	Repeat labeling, making sure to precipitate samples with 110µl of isopropanol per reaction.

Hybridization

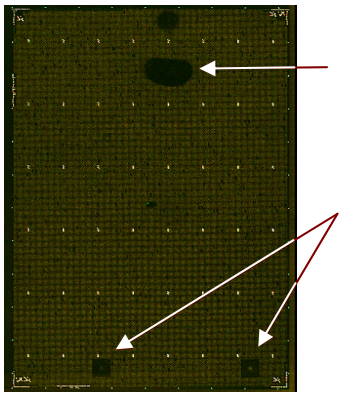
Problem	Possible Cause	Recommended Corrective Action
Mixer is poorly aligned on slide.	The slide was not flush in the PMAT.	Remove the mixer using the Mixer Disassembly Tool then reassemble using a new mixer.
	PMAT is not properly calibrated (multiple mixers misalign).	Send PMAT to Roche NimbleGen for calibration.
Hybridization solution does not enter the hybridization chamber.	The pipette tip is not situated properly on the fill port.	Refer to page 23 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 23 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 22 for proper braying technique. Repeat hybridization.
	The mixer port seals/multi-port seals were not fully adhered to the mixer.	Refer to page 24 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 NimbleGen Microarray Dryer or other 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 recommended microarray dryer: the NimbleGen Microarray Dryer.
 		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. The microarray dryer should achieve a top speed of at least 1,400 rpm in a minimum of 0.8 seconds.
Part of the array is missing from the array image.	The scan area is not specified properly.	Refer to page 33 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 scanner's documentation (or page 35 of this *User's Guide* if using the MS 200 scanner) 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 20) and the NimbleGen Hybridization System is set to and maintaining 42°C. Repeat hybridization. Refer to page 25 for proper washing technique. Repeat hybridization.
	Sample leaked out of the mixer during hybridization due to incomplete braying.	Refer to page 22 for proper braying technique. Repeat hybridization.
	Cy dye(s) are degraded due to exposure to light, ozone, and/or humidity.	Store primers at -20°C, protected from light. Maintain ozone levels below 20 ppb and humidity levels below 40%. Repeat hybridization.

Problem	Possible Cause	Recommended Corrective Action
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.
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 20) and the NimbleGen Hybridization System is set to and maintaining 42°C. Repeat hybridization. Refer to page 25 for proper washing technique. Repeat hybridization.
	Gain or PMT settings are not adjusted correctly.	Refer to the scanner's documentation (or page 35 of this *User's Guide* if using the MS 200 scanner) 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 22 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 page 25 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 20) and the NimbleGen Hybridization System is set to and maintaining 42°C. Repeat hybridization. Refer to page 25 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 scanner's documentation (or page 35 of this *User's Guide* if using the MS 200 scanner) 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.
Red and green features appear out of alignment.	Image has not been gridded.	Standard procedure is to grid each image (Cy3 and Cy5) individually using NimbleScan software.

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 22 for proper braying technique. ■ Excess sample is removed from around the loading ports. Refer to page 24 for proper sealing technique. ■ The mixer port seals/multi-port seals were not fully adhered to the mixer after loading sample. Refer to page 24 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 *Chapter 2. Sample Preparation & QC* (page 13). 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 20) and the NimbleGen Hybridization System is set to and maintaining 42°C. Repeat hybridization. Refer to page 25 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 room temperature 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 *Chapter 2. Sample Preparation & QC* (page 13). Repeat labeling and hybridization.
	Hybridization and/or wash conditions are not stringent enough.	Check that the hybridization solution was prepared correctly (refer to page 20) and the NimbleGen Hybridization System power is set to and maintaining 42°C. Repeat hybridization. Refer to page 25 for proper washing technique. Repeat hybridization.

Problem	Possible Cause	Recommended Corrective Action
NimbleScan fails to open the image TIFF file or Multiplex images fail to burst.	The wrong .ncd file was specified.	Refer to page 39 for instructions on how to specify the correct .ncd file when bursting multiplex arrays.
	The image was corrupted.	Rescan array.
	A non-16-bit TIFF image was specified.	Rescan and save as a 16-bit TIFF image. NimbleScan software reads only 16-bit grayscale images.
Multiplex images were burst incorrectly.	The entire array area was not scanned.	Refer to page 33 for instructions on how to specify the scan area. Check the image using the scanner software and rescan.
	The scanned area is too large.	Refer to page 33 for instructions on how to specify the scan area. Crop the image in NimbleScan software and attempt bursting again.
	The array area is not centered in the scanned image.	Refer to page 33 for instructions on how to specify the scan area. Crop the image in NimbleScan software or rescan if necessary.
The auto align function fails to grid the array image.	Fiducial features are dim.	Perform a manual alignment as described in the *NimbleScan Software User's Guide*.
	NimbleScan v2.4 or later was not used.	Install the latest version of NimbleScan software. Reload the image and perform the auto align function.
The auto align function improperly grids the array.	Bright artifacts are present in the corners of the array image.	Perform a manual alignment as described in the *NimbleScan Software User's Guide*.

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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D. Any action by Customer against Roche NimbleGen for Roche NimbleGen's breach of this warranty must be commenced within 12 months following the date of such breach. Notwithstanding such 12-month period, within twenty (20) days of the delivery of Data and/or Products to Customer, Customer must notify Roche NimbleGen in writing of any nonconformity of the Services and Products, describing the nonconformity in detail; otherwise all Services and Products shall be conclusively deemed accepted without qualification.

2. FURTHER LIABILITY LIMITATION

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

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