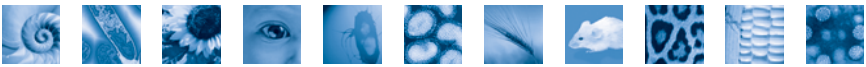
NimbleGen Arrays User's Guide

CGH Analysis

For life science research only.



HIGH - DEFINITION GENOMICS

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CGH Analysis

For life science research only.

Editions
Version 1.0, 16 Jan 2007; Version 2.0, 7 Sep 2007; Version 3.0, 31 Mar 2008;
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Chapter 1. Before You Begin

This user's guide describes the protocol for comparative genomic hybridization (CGH) using these NimbleGen array formats:

- 385K (385,000 probes)
- 4x72K (4 x 72,000 probes)
- 2.1M (2.1 million probes)
- 3x720K (3 x 720,000 probes)
- 12x135K (12 x 135,000 probes)

The CGH protocol involves independently labeling of test and reference genomic DNA using a NimbleGen Dual-Color DNA Labeling Kit and co-hybridization of these DNAs to a NimbleGen CGH array using a NimbleGen Hybridization System. Following hybridization, the arrays are washed, dried, and scanned. Array CGH data are extracted and analyzed using our NimbleScan software and SignalMap software.

What's New?

Version 5.0 of this user's guide includes instructions on using NimbleGen 3x720K arrays for CGH experiments.

Version 5.1 includes instructions for processing samples using the NimbleGen Dual-Color DNA Labeling Kit and NimbleGen Microarray Dryer.

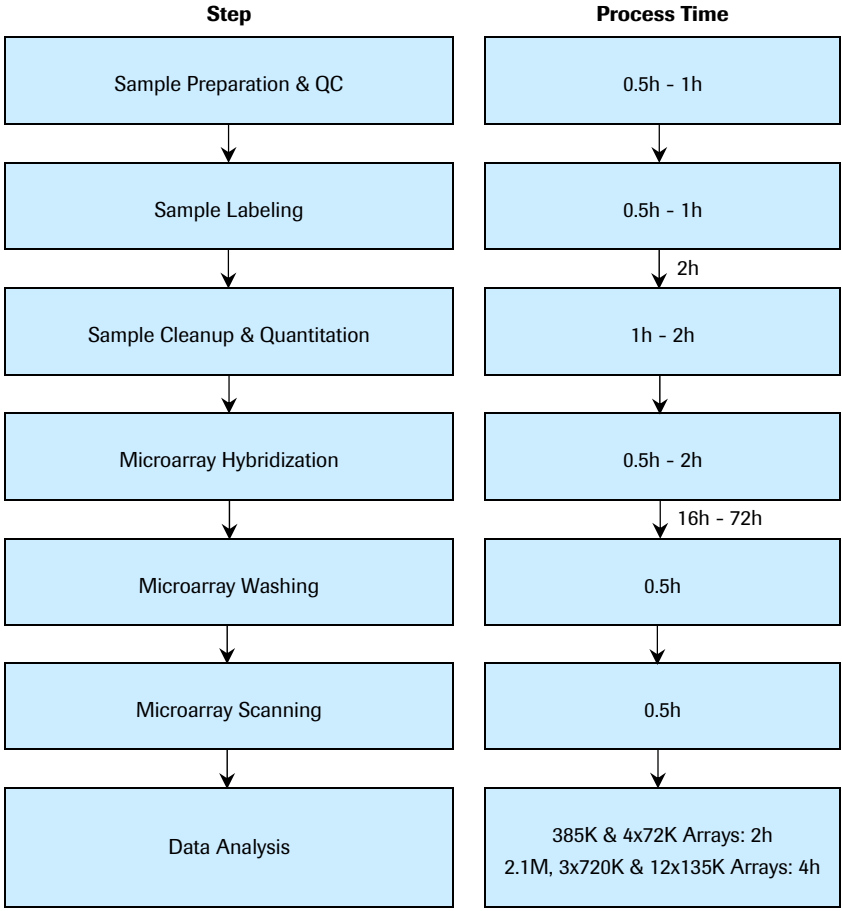


Figure 1: Workflow for NimbleGen CGH Analysis. Steps in the process and estimated time for each step, based on the processing of one slide, are shown in the boxes. (Note that a 12x135K array requires the longer processing time due to the handling of a larger number of samples.) Incubation times are indicated beneath the appropriate process times.

Components Supplied

Component	385K Array	4x72K Array	2.1M Array	3x720K Array	12x135K Array
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	One HX12 mixer is provided per 12x135K array
Mixer Port Seals or Mixer Multi-port Seals	Mixer port seals are provided with X1 mixers	Mixer multi-port seals are provided with X4 mixers	Mixer port seals are provided with HX1 mixers	Mixer port seals are provided with HX3 mixers	Mixer multi-port seals are provided with HX12 mixers
NimbleGen Arrays User's Guide: CGH Analysis			One booklet		
NimbleGen Design Files			One CD/DVD		

Microarray Storage

Store NimbleGen microarrays in a desiccator at room temperature until ready to 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 immediately after use.
- Cy dyes are ozone sensitive. Take the necessary precautions to keep atmospheric ozone levels below 20ppb (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.
- Roche NimbleGen recommends using a NanoDrop Spectrophotometer for quantifying and characterizing nucleic acid samples because this instrument requires only 1.5µl 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 Hybridization System

Choose between the following:

Equipment	Supplier	Process Quantity	Item Number
NimbleGen Hybridization System 4*	Roche NimbleGen	4 slides	05223652001 (110V)
			05223679001 (220V)
NimbleGen Hybridization System 12*	Roche NimbleGen	12 slides	05223687001 (110V)
			05223695001 (220V)

* 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.

Microarray Drying System

Equipment	Supplier	Process Quantity	Item Number
NimbleGen Microarray Dryer	Roche NimbleGen	24 slides	05223636001 (110V)
			05223644001 (220V)

Scanner

Equipment	Supplier	Item Number
GenePix 4000B	Molecular Devices	NA

Software

Program	Supplier	Item Number
NimbleScan v2.5	Roche NimbleGen	05225035001 (Individual License)
		05225043001 (Site License)
SignalMap v1.9	Roche NimbleGen	05225051001 (Individual License)

Standard Laboratory Equipment

Equipment	Supplier	Item Number
NimbleGen Compressed Gas Nozzle	Roche NimbleGen	05223628001
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 and 12x135K arrays)	Gilson	F148501
Microman M100 Pipette (recommended for 385K, 2.1M, and 3x720K arrays)	Gilson	F148504
Sonicator with converter, ½" externally threaded disruptor horn, and tapered microtips	Branson	Sonicator: Model 450 Sonifier Converter: 101-135-022 Disruptor Horn: 101-147-037 Microtips: 101-148-062
Spectrophotometer	NanoDrop	ND-1000

Equipment	Supplier	Item Number
SpeedVac	Thermo Savant	
Thermocycler	*Multiple Vendors*	
Vortex Mixer	*Multiple Vendors*	

Consumables & Accessories from Roche NimbleGen

Component	Package Size / Process Quantity	Item Number
NimbleGen X1 Mixer (for 385K arrays; includes mixer port seals)	5 mixers	05391717001
	10 mixers	05223725001
NimbleGen X4 Mixer (for 4x72K arrays; includes mixer multi-port seals)	5 mixers	05391725001
	10 mixers	05223733001
NimbleGen HX1 Mixer (for 2.1M arrays; includes mixer port seals)	5 mixers	05391733001
	10 mixers	05223741001
NimbleGen HX3 Mixer (for 3x720K arrays; includes mixer port seals)	5 mixers	05391741001
	10 mixers	05223750001
NimbleGen HX12 Mixer (for 12x135K arrays; includes mixer multi-port seals)	5 mixers	05391768001
	10 mixers	05223768001
NimbleGen Dual-Color DNA Labeling Kit	10 Cy3 labeling reactions and 10 Cy5 labeling reactions	05223547001
NimbleGen Hybridization Kit	■ 100 hybridizations using X1 mixers ■ 160 hybridizations using X4 mixers ■ 40 hybridizations using HX1 mixers ■ 100 hybridizations using HX3 mixers ■ 120 hybridizations using HX12 mixers	05223474001

Component	Package Size / Process Quantity	Item Number
NimbleGen Hybridization Kit, LS (Large Scale)	■ 300 hybridizations using X1 mixers ■ 480 hybridizations using X4 mixers ■ 120 hybridizations using HX1 mixers ■ 300 hybridizations using HX3 mixers ■ 480 hybridizations using HX12 mixers	05223482001
NimbleGen Sample Tracking Control Kit	■ 120 hybridizations using 4x72K or 12x135K arrays ■ 300 hybridizations using 3x720K arrays	05223512001
NimbleGen Wash Buffer Kit	10 washes (processing 4 slides per wash)	05223504001
NimbleGen Array Processing Accessories		05223512001

Contents of NimbleGen Kits & Accessories

Kit	Contents
NimbleGen Dual-Color DNA Labeling Kit	■ 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-) 50U/µl (vial 5) ■ 10mM dNTP Mix (vial 6) ■ Stop Solution (0.5M EDTA) (vial 7) ■ 5M NaCl (vial 8)
NimbleGen Hybridization Kit and NimbleGen Hybridization Kit, LS	■ 2X Hybridization Buffer ■ Hybridization Component A ■ Alignment Oligo[1]
NimbleGen Sample Tracking Control Kit	Sample Tracking Controls[2]

Kit	Contents
NimbleGen Wash Buffer Kit	■ 10X Wash Buffer I ■ 10X Wash Buffer II ■ 10X Wash Buffer III ■ 1M DTT
NimbleGen Array Processing Accessories	■ Slide Rack ■ Wash Tanks ■ Slide Containers

1 The Alignment Oligo is a mixture of Cy3- and Cy5-labeled 48mer 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 48mer 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 from Other Vendors

Component	Supplier	Package Size	Item Number
β-Mercaptoethanol	Sigma Aldrich	25ml	M3148
Absolute Ethanol	Sigma Aldrich	500ml	E702-3
Compressed Inert Gas*	*Multiple Vendors*		
CP10 Pipette Tips (for 4x72K and 12x135K 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	500ml	I-9516
Water: reagent grade, ACS, nonsterile, type 1	VWR	2.5 gallon	RC915025
Cotton Swabs	*Multiple Vendors*		

* Roche NimbleGen recommends using the NimbleGen Compressed Gas Nozzle to gently blow compressed inert 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 Roche NimbleGen Account Manager or Roche Microarray Technical Support. Go to *www.nimblegen.com/arraysupport* for contact information.

Notes

Chapter 2. Sample Preparation & QC

Chapter 2 describes sample requirements and preparation and the sample QC protocol for NimbleGen array CGH experiments.

Sample Requirements

- Purified, unamplified, and unfragmented genomic DNA (gDNA) is required for optimal sample labeling and hybridization.
- Roche NimbleGen recommends starting with the following gDNA amounts for each hybridization:

Sample Requirements	385K Array	Each Sample for a 4x72K Array	2.1M Array	Each Sample for a 3x720K Array	Each Sample for a 12x135K Array
Test gDNA	2µg	2µg	2.5µg	1.5µg	1.5µg
Reference gDNA	2µg	2µg	2.5µg	1.5µg	1.5µg

- Samples should be prepared at a concentration of 250ng/µl to 1,000ng/µl in nuclease-free water or 1X TE buffer (10mM Tris-HCl and 0.1mM EDTA, pH 7.5 - 8.0).
- Samples should have an $A_{260}/A_{280} \geq 1.8$ and $A_{260}/A_{230} \geq 1.9$ for optimal labeling yields.

Sample Preparation & QC

Note: *Roche NimbleGen has tested several common genomes and found that the sonication step described below can be omitted without adversely affecting CGH data quality.*

1. Dilute test and reference gDNA to 80µl with VWR water in a 1.5ml micro-centrifuge tube.
2. Clean the sonicator tip with 70% ethanol and wipe dry with a tissue.
3. Select the following settings on a Branson model 450 Sonifier:

   Note: *If using another sonicator, adjust the settings as necessary to produce a smear from ~500bp to 2,000bp.*

   - Time = 10 seconds
   - Amplitude = 10%
   - Pulse On = 0.5 second
   - Pulse Off = 0.5 second
4. Lower the sonicator tip to near the bottom of the tube and push the start button. Positioning of the probe near the bottom of the tube will prevent splashing and ensure complete sonication of your sample.

   Important: *Wear hearing protection when operating the sonicator.*
5. To QC your sample, run 250ng of gDNA on a NanoDrop Spectrophotometer and 250ng of pre- and post-sonicated gDNA on a 1% agarose gel to ensure they show no signs of RNA contamination or degradation.

   Important: *Genomic DNA should appear as a single prominent band greater than 12kb. If the sample appears as more than one band or as a smear, the DNA may be degraded or have a contaminant that could affect the labeling procedure. RNA contamination will result in a smear less than 200bp.*

Sonicated sample should appear as a smear from ~500bp to 2,000bp with the majority of the fragments migrating between 500bp and 1,000bp. Genomic DNA exhibiting significant degradation (all bands <500bp) is unsuitable for CGH analysis.

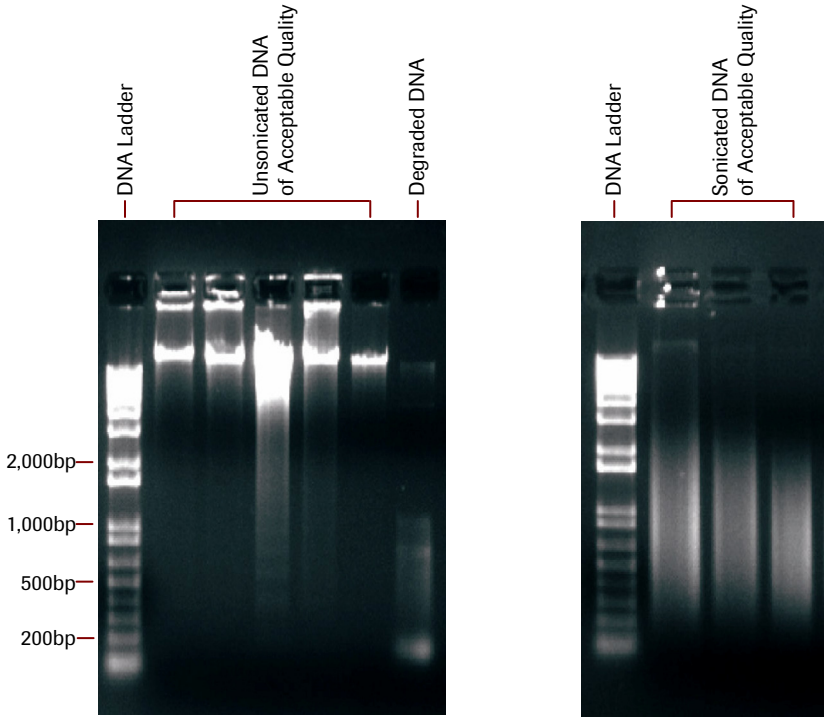


Figure 2: Examples of Agarose Gel Electrophoresis for Unsonicated DNA (left) and Sonicated DNA (right)

Notes

Chapter 3. Sample Labeling

Chapter 3 describes how to label your gDNA samples using a NimbleGen Dual-Color DNA Labeling Kit. Be aware of the following when using these kit contents:

- Aliquot dNTPs and Cy primers into single-use amounts.
- 5M NaCl could precipitate. Vortex if necessary.

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 test samples with Cy3 and reference samples with Cy5, although the dyes can be reversed if you choose.

1. Prepare the following solution:

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

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 462µl each of Random Primer Buffer with β-Mercaptoethanol. Aliquot to 40µl individual reaction volumes in 0.2ml thin-walled PCR tubes and store at -20°C, protected from light.

   Note: *Do not store diluted primers longer than 4 months.*

3. Assemble the test and reference samples in separate 0.2ml thin-walled PCR tubes.

Component	All Array Formats	
	Test Sample	Reference Sample
gDNA Sample prepared in Chapter 2	1µg*	1µg*
Diluted Cy3-Random Nonamers from step 2	40µl	
Diluted Cy5-Random Nonamers from step 2		40µl
Nuclease-free water (vial 1)	To volume (80µl)	To volume (80µl)
Total	**80µl**	**80µl**

***** If labeling sonicated gDNA, 2.1M arrays require two labeling reactions for both the test and reference samples per slide. To accomplish this, assemble two separate 0.2ml thin-walled PCR tubes each containing 1µg of gDNA of test and reference sample. Test and reference sample pairs intended for hybridization to the same 2.1M array should be labeled in parallel.

Note: *The NimbleGen standard protocol specifies 1µg of starting material in a 100µl reaction (step 6, below). For multiplex slides where less labeled DNA is required for hybridizations, it is possible to input less genomic DNA into the labeling reaction volume. For 4x72K, 3x720K, and 12x135K arrays, Roche NimbleGen recommends starting with 500ng of gDNA in a 100µl reaction.*

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

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

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

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

dNTP/Klenow Master Mix: Recipe per Sample	All Array Formats
10mM dNTP Mix (vial 6)	10µl
Nuclease-free Water (vial 1)	8µl
Klenow Fragment (3'->5' exo-) 50U/µl (vial 5)	2µl
Total	**20µl**

6. Add 20µl 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	
	Test Sample	Reference Sample
Reaction volume from step 4	80µl	80µl
dNTP/Klenow Master Mix from step 5	20µl	20µl
Total	**100µl**	**100µl**

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

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

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

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

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

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

11. Add 5M NaCl to each tube.

Component	All Array Formats	
	Test Sample	Reference Sample
Reaction volume from step 10	110µl	110µl
5M NaCl (vial 8)	11.5µl	11.5µl
Total	**121.5µl**	**121.5µl**

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

Component	All Array Formats	
	Test Sample	Reference Sample
Reaction volume from step 11	121.5µl	121.5µl
Isopropanol	110µl	110µl
Total	**231.5µl**	**231.5µl**

Note: Up to 4 reactions containing the same sample can be combined in a 1.5ml tube and precipitated together. If combined, be sure to scale the isopropanol volume appropriately.

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 500µl 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 SpeedVac 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 pellets in 25µl Nuclease-free Water (vial 1) per reaction. For 2.1M arrays, if you combined reactions, scale the volume accordingly.

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:

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

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

22. Based on the concentration, calculate the volume of the test sample and reference sample required for each hybridization per the following table and combine both test and reference samples in a 1.5ml tube:

Sample Requirements	385K Array	4x72K Array	2.1M Array	3x720K Array	12x135K Array
Test Sample	6µg	4µg	34µg	31µg	20µg
Reference Sample	6µg	4µg	34µg	31µg	20µg

23. Dry contents in a SpeedVac 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.

Notes

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.

   Note: *Be aware that the temperature of the Hybridization System may fluctuate during stabilization.*

2. Resuspend the dried sample pellet in VWR water (for 385K and 2.1M arrays) or appropriate Sample Tracking Control (for 4x72K, 3x720K, and 12x135K arrays) according to the following table. Each sample to be hybridized to a 4x72K, 3x720K, or 12x135K array should be resuspended in a unique STC. Record which STC is used for each sample.

Component	385K Array	Each Sample for a 4x72K Array	2.1M Array	Each Sample for a 3x720K Array	Each Sample for a 12x135K Array
Reagent for resuspension	VWR Water	Sample Tracking Control	VWR Water	Sample Tracking Control	Sample Tracking Control
Volume to add to Cy-labeled Sample from step 23 in Chapter 3	5µl	3.3µl	12.3µl	5.6µl	3.3µl

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, 3x720K, and 12x135K arrays, the amount listed is sufficient to hybridize all 4, 3, or 12 subarrays, respectively, on a 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	12x135K Array
2X Hybridization Buffer	11.8µl	29.5µl	29.5µl	35µl	88.5µl
Hybridization Component A	4.7µl	11.8µl	11.8µl	14µl	35.4µl
Alignment Oligo	0.5µl	1.2µl	1.2µl	1.4µl	3.6µl
Total	**17µl**	**42.5µl**	**42.5µl**	**50.4µl**	**127.5µl**

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	Each Sample Pair for a 12x135K Array
Resuspended sample from step 2	5µl	3.3µl	12.3µl	5.6µl	3.3µl
Hybridization solution from step 4	13µl	8.7µl	31.7µl	14.4µl	8.7µl
Total	**18µl**	**12µl**	**44µl**	**20µl**	**12µl**

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.

   Note: *For best results, use the NimbleGen Compressed Gas Nozzle to gently blow compressed inert 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
12x135K array	HX12 mixer

2. Position the Precision Mixer Alignment Tool (PMAT) with its hinge on the left. Open the PMAT (Figure 3).

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 3).

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 inert gas across the mixer and slide to remove dust.

   Note: *Take care to align the slide correctly in the PMAT. Incorrectly aligned slides may result in inaccurate attachment of the mixer and 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.

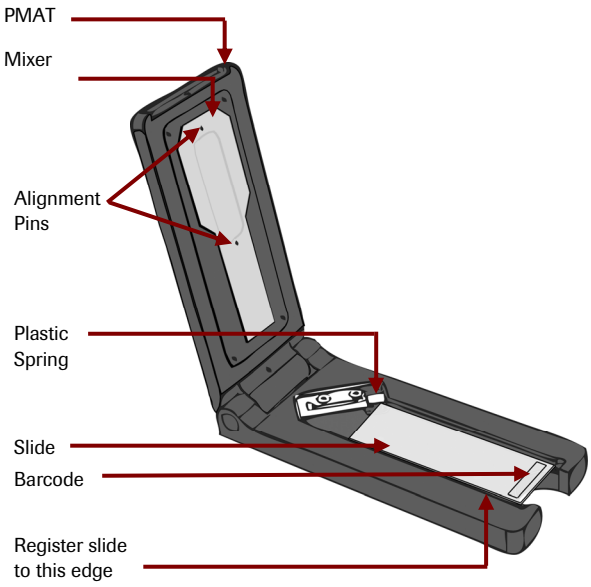


Figure 3: PMAT with X1 Mixer and Slide

7. Remove the mixer-slide assembly from the PMAT.
8. Place the mixer-slide assembly on the back of a 42°C heating block 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, HX3, and HX12 mixers, first use a corner of the Mixer Brayer to rub the borders between the subarrays and then rub around the outside of the subarrays. 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:

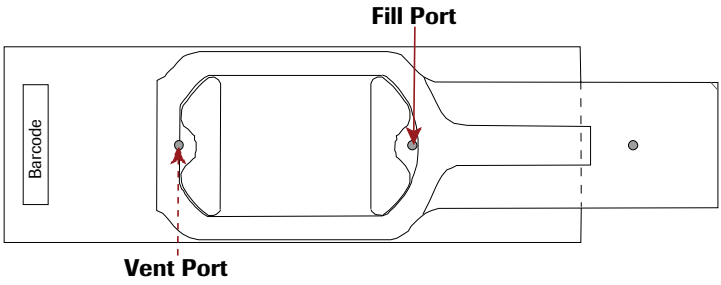


Figure 4: X1 Mixer and Slide for a 385K Array

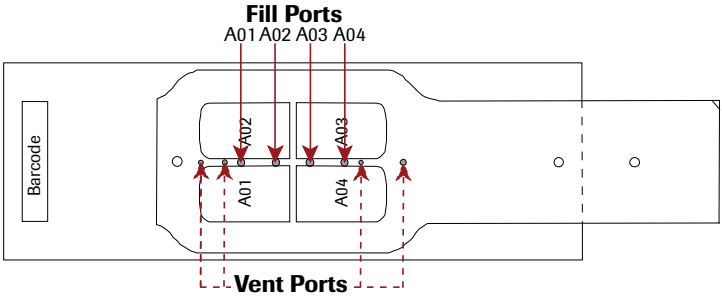


Figure 5: X4 Mixer and Slide for a 4x72K Array

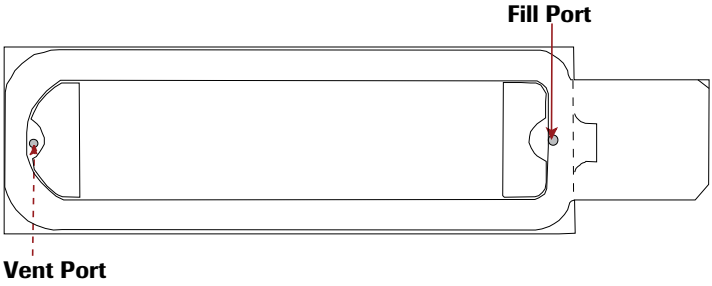


Figure 6: HX1 Mixer and Slide for a 2.1M Array

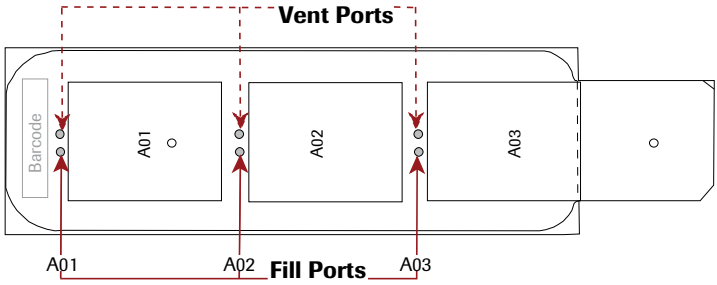


Figure 7: HX3 Mixer and Slide for a 3x720K Array

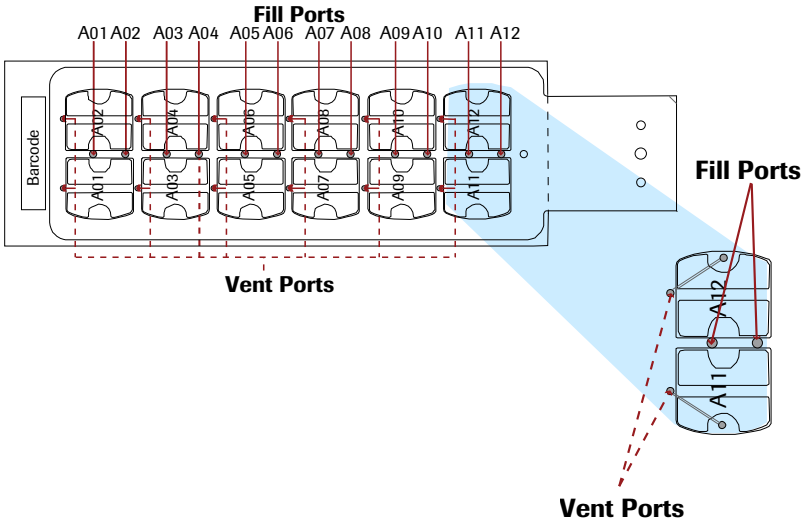


Figure 8: HX12 Mixer for a 12x135K Array. The enlarged image shows the positioning of fill and vent ports on the HX12 mixer.

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	12x135K Array
Sample Loading Volume	16µl	9µl	41µl	18µl	6µl
Pipette Tip	CP100	CP10*	CP100	CP100	CP10*

* 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.

- For 12x135K arrays:
    a. Load sample into the A01 fill port. Due to the close proximity of the fill and vent ports, do not overfill the arrays. Load sample until it enters the vent port channel. Do not allow sample to come to the surface of the HX12 mixer. If overflow occurs, dry the overflow from the fill and vent ports with a cotton swab after loading. Repeat loading sample into the A02 - A06 fill ports, using a fresh cotton swab for drying the ports for each array.
    b. Use one mixer multi-port seal to cover A01 - A06 fill ports. Apply light pressure at each fill port to seal.
    c. Use one mixer multi-port seal to cover A01, A03, A05 vent ports and one mixer multi-port seal to cover A02, A04, A06 vent ports. Apply light pressure at each vent port to seal.
    d. Repeat steps a - c to load samples into and seal A07 - A12.

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 the following incubation times:
    - For 385K and 4x72K arrays: 16 - 20 hours
    - For 2.1M arrays: 60 - 72 hours
    - For 3x720K and 12x135K arrays: 40 - 72 hours

    Roche NimbleGen has found that longer hybridization times result in higher signal-to-noise ratios, which may be beneficial for some experiments.

Step 4. Wash Hybridized Arrays

Important: *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 7).
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	225ml	225ml
10X Wash Buffer I, II, or III	25ml	25ml
1M DTT	25µl	25µl
Total	**250ml**	**250ml**

Washing One Slide	Wash I (user-supplied dish[1])	Washes I, II, and III (slide container[2])
VWR Water	225ml	22.5ml
10X Wash Buffer I, II, or III	25ml	2.5ml
1M DTT	25µl	2.5µl
Total	**250ml**	**25ml**

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.

   Important: *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.

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

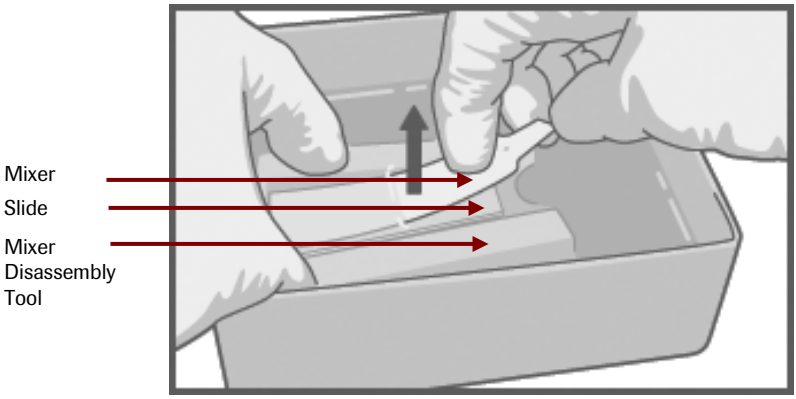


Figure 9: Using the Mixer Disassembly Tool to Remove a Slide from a Mixer

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.

Note: *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 10) 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 submerged at all times during all wash steps.

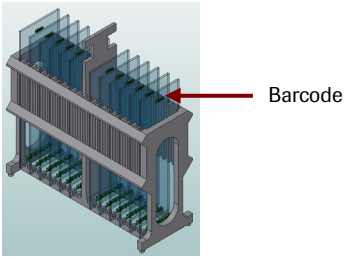


Figure 10: Insert Slides with the Barcode at the Top into the Slide Rack

Note: *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.

Important: *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 remove excessive 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).

Important: *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.

    Note: *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 a GenePix 4000B Scanner and associated software. Keep arrays in a dark desiccator until you are ready to scan them.

1. Turn on the scanner using the power switch on the back right side.
2. Launch the GenePix software 10 minutes before scanning to allow lasers to warm.
3. Open the scanner door and the slide carriage.
4. Using a NimbleGen Compressed Gas Nozzle, gently blow compressed inert gas across the slide to remove any dust or debris from the array. Do not use canned aerosol compressed air for this purpose.
5. Place the slide in the slide carriage so that the array is face down and the barcode end is closest to you.
6. Move the black lever on the left side of the carriage to the left until the slide is lying flat in the carriage.
7. Release the lever so that the slide is gently pushed to the right side of the carriage and held firmly.
8. Close the carriage (you should hear a click) and slide the scanner door shut.
9. Open the Hardware Settings dialog box (right side, second icon from the bottom or Alt + Q). Select the following settings for scanning:

   - Wavelength (532 = Cy3, 635 = Cy5)
   - 532 PMT Gain = 650
   - 635 PMT Gain = 750
   - Power (%) = 100%
   - Pixel size (µm) = 5
   - Lines to average = 1
   - Focus position (µm) = 0

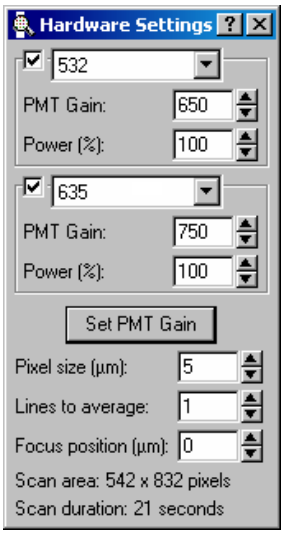


Figure 11: Hardware Settings Dialog Box

10. Under **Image** on the left side of the screen, select **Ratio** to view both channels simultaneously.

11. Preview the entire slide by clicking the preview scan icon (or Alt + P). This will allow you to locate the array(s) on the slide.

12. Under **Tools** on the left side of the screen, click the scan area icon (or Ctrl + V). Click and drag to define a box that surrounds the array image on 385K arrays, all 4 subarray images on 4x72K arrays, all 3 subarray images on 2.1M arrays, or all 12 subarray images on 12x135K arrays. The box dimensions should be stretched to create a box just slightly larger than the array image(s). Balance the border around the scanning area to ensure proper data extraction later. It is critical to include all of the corner probes within the scan image.

Note: Only one scan is required per slide. It is not necessary to scan each subarray individually. Be sure to scan the entire slide regardless of whether each subarray was used.

Approximate coordinates and size of the scan area are as follows and can be entered into the Scan Area Properties (F10):

Coordinate	385K and 4x72K Arrays	2.1M, 3x720K, and 12x135K Arrays
Left	4,200	2,600
Top	26,000	2,800
Width	13,400	15,500
Height	18,000	62,200

13. Scan the array by clicking the play icon (or Alt + D).

Switch to zoom mode by clicking on the array image and pressing Z on the keyboard. Click and drag the cursor over the region in which you want to zoom. To restore the prior view, press Alt + Z or use the zoom icon. Center the entire array image in the viewing window.

14. Based on the appearance of the array features, adjust the PMT setting as appropriate (you can make this adjustment while the scan is running). The features should be mostly yellow. If the features are mostly green, either decrease the 532 PMT setting or raise the 635 PMT setting. If the features are mostly red or orange, either raise the 532 PMT setting or decrease the 635 PMT setting.

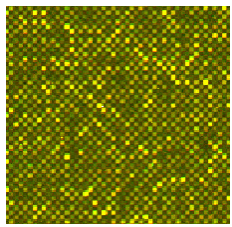


Figure 12: Example Array Features

15. Zoom into a region scanned under the most recent PMT settings. Zoom into as large a region as possible to get an accurate reading for the whole array. If there is high background outside of the array area, avoid these regions. The histogram is calculated from all regions bound by the current view, but ratios must be selected for array areas only.

16. Click the Histogram tab at the top of the left side of the screen to check the global intensity of the features.

    - On the top left side of the screen, make sure the *Wavelength 532* and *Wavelength 635* boxes are checked so both wavelength histograms are displayed.
    - On the left side of the screen, under Y Axis, make sure the *Log Axis* box is checked.

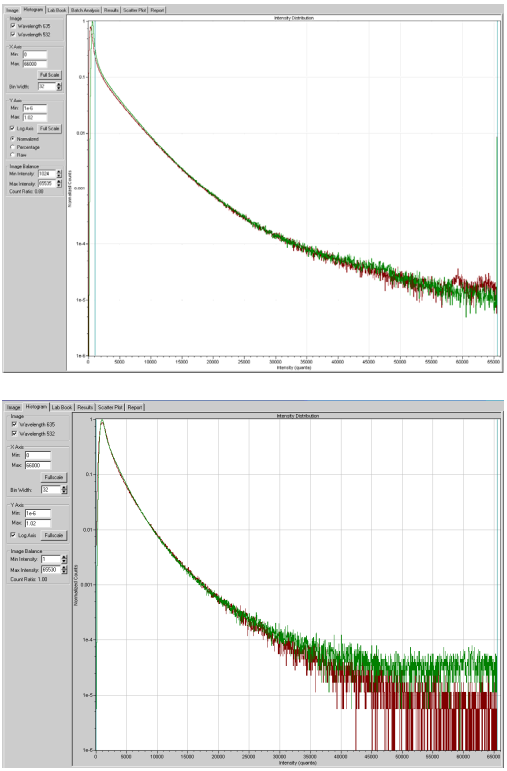


Figure 13: Example Histograms. The top histogram shows perfectly overlapping curves from a scanned image with properly adjusted red and green PMT levels. The bottom histogram shows an acceptable image. Note that the curves are overlapping for nearly the entire intensity range of the image.

- The red and green curves should be superimposed or as close as possible to one another. If the red curve is above the green, lower the red PMT setting or raise the green PMT setting on the *Image* tab.
- The curve should end above 1e-5 normalized counts at the 65,000 intensity level (saturation).
- The superimposed position of the two curves is more important than obtaining the 1e-5 normalized counts.
- The histogram graphs the region of the image viewable on-screen in the image tab. If the histogram is no longer changing, either the scan is complete or the area on the current view has been scanned.

17. After the PMT settings are properly adjusted, stop the current scan, and do not save this image. Restart the scan and allow the scan to run completely at the new settings.

18. When the scan completes, zoom into each corner and confirm all features are visible. If some features are missing, refer to step 12. Adjust the scanning area appropriately and rescan.

19. Save the images for the 532nm and 635nm wavelengths using the following naming convention:

NNNNN_XXXXX_WWW.tif

where **NNNNN** is the slide's barcode number, **XXXXX** is optional user-defined text, and **WWW** is the wavelength.

***Important**: Be sure to save both the 532nm and 635nm images as single image .tif files. You will need both images to calculate test versus reference ratios.*

20. (For 4x72K, 3x720K, or 12x135K arrays only) If the signal intensities between the subarrays are not uniform, you can scan the slide multiple times, each time adjusting the PMT gain to acquire the best image of a specific subarray. Alternatively, scan and save each subarray individually (refer to page 39).

Scan and save the images as described in steps 17 - 19, specifying the subarray for which the PMT gain was optimized in the file name.

***Note:** Non-uniformity in the signal intensities between arrays will be corrected when normalization is applied during analysis using NimbleScan software.*

21. After scanning, remove the slide from the scanner. Store slides in a dark desiccator in the event that rescanning is necessary.

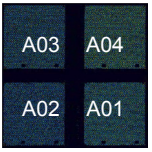
Scanning Individual Subarrays on Multiplex Arrays

If the signal intensities between subarrays on 4x72K, 3x720K, or 12x135K (multiplex) arrays are not uniform, you can scan individual subarray(s) with a higher or lower PMT gain, as appropriate, to acquire the best image of the subarray(s). The locations of individual subarrays on the scanned image are shown in Figure 14. The coordinates in the associated tables may be used for defining the scan area for an individual subarray.

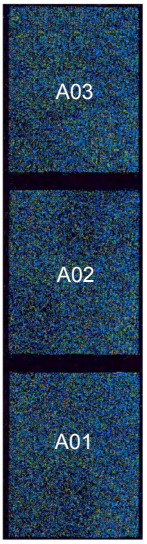
Scan and save the image as described in steps 17 - 19 above, specifying the subarray and the PMT gain in the file name.

***Important**: When viewing this scanned image in NimbleScan software, you do not need to burst (separate) its file as described in Chapter 6.*

4x72K Array	Left	Top	Width	Height
A01	11,540	36,075	6,100	8,340
A02	4,100	36,075	6,100	8,340
A03	4,100	26,420	6,100	8,340
A04	11,540	26,420	6,100	8,340



3x720K Array	Left	Top	Width	Height
A01	2,800	44,600	15,500	20,000
A02	2,800	23,200	15,500	20,000
A03	2,800	2,100	15,500	20,000



12x135K Array	Left	Top	Width	Height
A01	11,250	54,950	6,850	9,200
A02	3,175	54,950	6,850	9,200
A03	11,250	44,500	6,850	9,200
A04	3,175	44,500	6,850	9,200
A05	11,250	33,700	6,850	9,200
A06	3,175	33,700	6,850	9,200
A07	11,250	23,300	6,850	9,200
A08	3,175	23,300	6,850	9,200
A09	11,250	12,600	6,850	9,200
A10	3,175	12,600	6,850	9,200
A11	11,250	2,150	6,850	9,200
A12	3,175	2,150	6,850	9,200

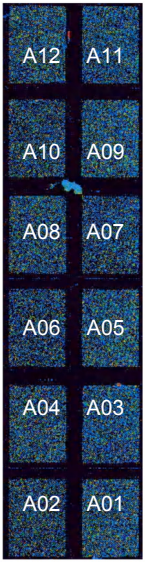


Figure 14. Dimensions and Example Images of Multiplex Arrays

Chapter 6. NimbleScan Data Analysis

Chapter 6 describes how to import your scanned image and extract the data using NimbleScan software. Refer to the *NimbleScan v2.5 User's Guide* for computer system requirements and detailed information on using the software.

Step 1. Burst Multiplex Image (4x72K, 3x720K & 12x135K Arrays Only)

If your files contain the scanned images of 4x72K, 3x720K, or 12x135 arrays, follow the steps below to burst (separate) each slide image into 4, 3, or 12 separate array images, respectively. 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) in the Design File disk provided with the 4x72K, 3x720K, or 12x135K 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 12 image files. The subarray designation (A01 - A04 for 4x72K, A01 - A03 for 3x720K, and A01 - A12 for 12x135K arrays) for each bursted image is specified in the original image's file name. For example:
   - **NNNNN**A01_**XXXXX**_**WWW**.tif…**NNNNN**A04_**XXXXX**_**WWW**.tif
   - **NNNNN**A01_**XXXXX**_**WWW**.tif…**NNNNN**A03_**XXXXX**_**WWW**.tif
   - **NNNNN**A01_**XXXXX**_**WWW**.tif…**NNNNN**A12_**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.

   Note: *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.

   Note: *Open and grid both the 532 and 635 images separately.*

2. Navigate to the directory containing your array images and select one image. If you scanned multiplex arrays at multiple PMT gain settings, remember to select the optimized image of each array for further analysis.
3. Select the design file (.ndf) in the Design File 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.

   Note: *If the software indicates auto alignment was not successful, perform a manual alignment as described in the NimbleScan v2.5 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.

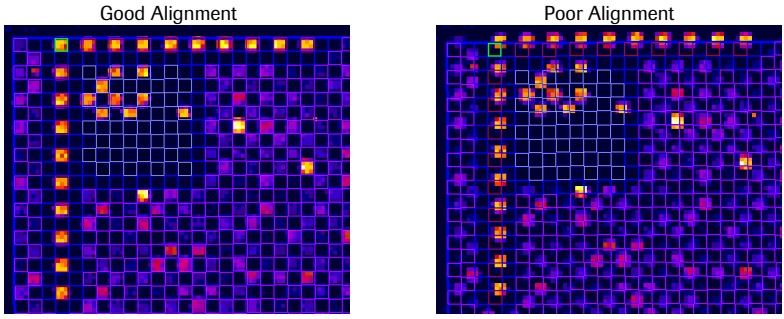


Figure 15: Examples Showing Good and Poor Grid Alignments

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 and 2.1M 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, 3x720K, or 12x135K array, proceed to "Step 4. Confirm Experimental Integrity (4x72K, 3x720K, and 12x135K 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 & 12x135K Arrays Only)

A unique STC should have been added to each test and reference sample pair prior to loading onto 4x72K, 3x720K, or 12x135K 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 16). Roche NimbleGen recommends performing a sample tracking analysis (refer to page 45) and visually checking the STC features along the perimeter (refer to page 47) to confirm that the correct sample has been added to each array.

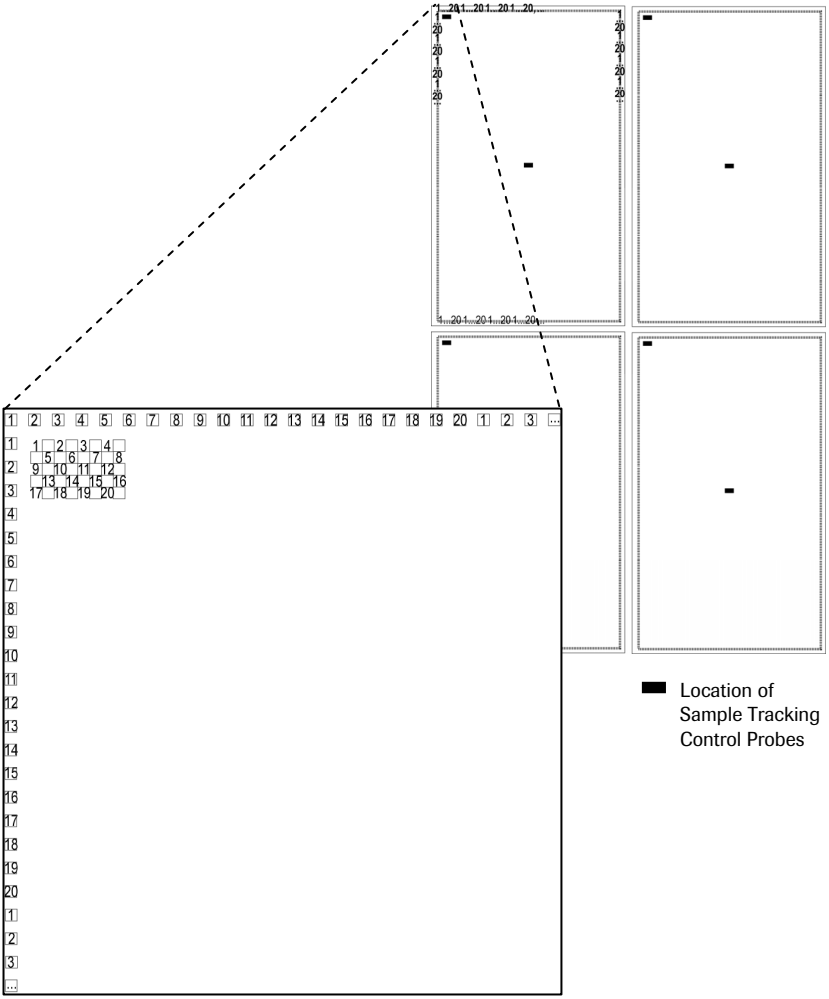


Figure 16: Location and Numbering of Sample Tracking Control Probes on a 4x72K Array

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 (Figure 16). 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 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 17.
    a. Confirm that the Image ID, Design ID, and Design Name are accurate.
    b. 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.
    c. 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.
    d. 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.
    e. 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	193	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 17: 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 18 shows an example of an array hybridized with a sample containing STC 5.

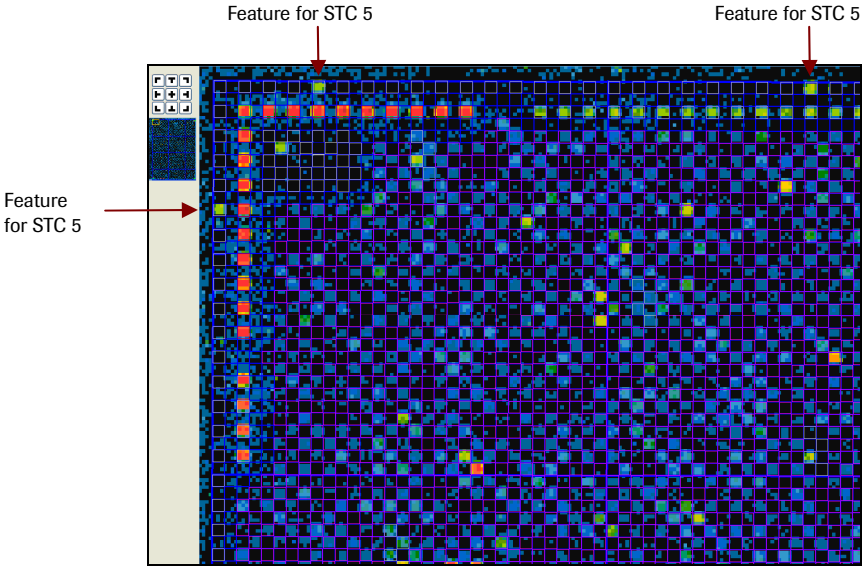


Figure 18: Example of an Array Hybridized with Sample Containing STC 5

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 v2.5 software.

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

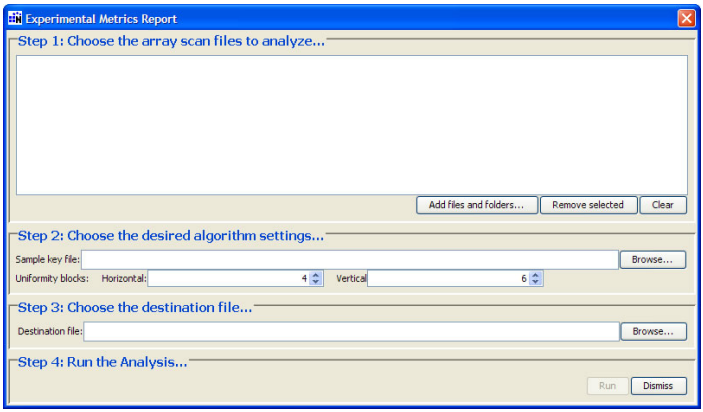


Figure 19: Experimental Metrics Report Dialog Box

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 12 images for 12x135K 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, etc.), 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 20.

IMAGE_NAME	INTERQUARTILE_DENSITY	RATIO_RANGE	SIGNAL_RANGE	UNIFORMITY_MEAN	UNIFORMITY_CV	NUM_EMPTY	MEAN_EMPTY	NUM_EXPERIMENTAL	MEAN_EXPERIMENTAL	NUM_RANDOM	MEAN_RANDOM
080310A01_532.tif	1.076	0.109	0.208	7441.633	0.048	146414	3688.3	141000	6895.972	0	0
080310A01_635.tif	1.491	0.109	0.277	8964.27	0.051	146414	3385.3	141000	8255.4	0	0
080310A02_532.tif	0.978	0.168	0.191	7061.371	0.037	146414	3673.8	141000	6595.462	0	0
080310A02_635.tif	1.472	0.168	0.207	8628.102	0.033	146414	3045.3	141000	7880.46	0	0
080310A03_532.tif	1.048	0.101	0.168	7639.469	0.03	146414	3700.4	141000	7075.255	0	0
080310A03_635.tif	1.469	0.101	0.214	8563.141	0.042	146414	3375.4	141000	7968.705	0	0
080310A04_532.tif	0.937	0.124	0.132	6388.964	0.024	146414	3206	141000	5932.882	0	0
080310A04_635.tif	1.465	0.124	0.195	7985.767	0.033	146414	2873.3	141000	7297.637	0	0
080310A05_532.tif	1.025	0.13	0.177	7007.113	0.042	146414	3612.1	141000	6553.059	0	0
080310A05_635.tif	1.485	0.13	0.261	8256.388	0.046	146414	3187.1	141000	7633.495	0	0
080310A06_532.tif	0.953	0.168	0.181	5955.887	0.033	146414	3036.6	141000	5555.164	0	0
080310A06_635.tif	1.486	0.168	0.265	7647.36	0.045	146414	2755.6	141000	7008.088	0	0
080310A07_532.tif	0.86	0.157	0.168	5393.128	0.033	146414	2934.3	141000	5057.163	0	0
080310A07_635.tif	1.453	0.157	0.304	7113.597	0.042	146414	2638.9	141000	6516.516	0	0
080310A08_532.tif	1.107	0.146	0.178	7075.086	0.033	146414	3293.7	141000	6531.669	0	0
080310A08_635.tif	1.506	0.146	0.287	7776.009	0.04	146414	2952.8	141000	7168.038	0	0
080310A09_532.tif	0.88	0.184	0.533	6128.743	0.089	146414	3473.9	141000	5828.778	0	0
080310A09_635.tif	1.284	0.184	0.639	7290.934	0.096	146414	3067.9	141000	6766.15	0	0
080310A10_532.tif	0.897	0.185	0.709	6503.046	0.115	146414	3620.1	141000	6124.507	0	0
080310A10_635.tif	1.244	0.185	0.82	6883.843	0.098	146414	3242.8	141000	6557.719	0	0
080310A11_532.tif	0.214	0.462	0.249	1610.62	0.039	146414	1534.9	141000	1629.268	0	0
080310A11_635.tif	0.553	0.462	0.273	297.023	0.044	146414	295.68	141000	296.689	0	0
080310A12_532.tif	0.198	0.219	0.157	1654.929	0.03	146414	1589.8	141000	1673.642	0	0
080310A12_635.tif	0.542	0.219	0.203	298.135	0.036	146414	297.84	141000	298.06	0	0

Figure 20: 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, 3x720K, or 12x135K arrays), calculating the average $\log_2$ ratio of each block and calculating the range from the block averages.

  Note: *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.

Step 6. (Optional) Create Pair Reports

Important: *In previous versions of NimbleScan software, .pair files were used as input for copy number analysis. Using NimbleScan v2.5, copy number analysis (Step 7) can use either .pair or gridded .tif files (generated in Step 3) as input.*

Pair reports (.pair), the raw data format for NimbleGen CGH 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**.

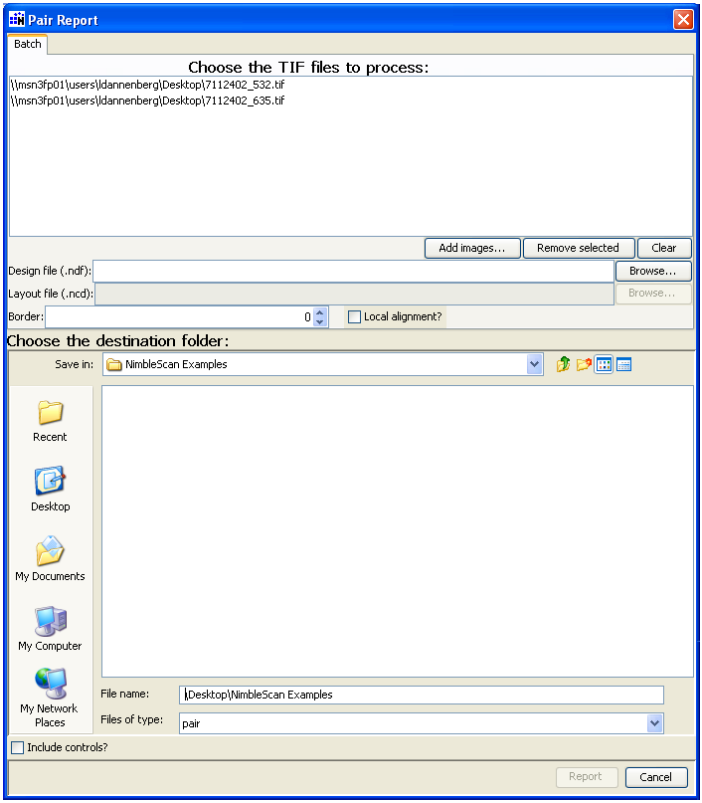


Figure 21: Pair Report Dialog Box

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:

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

   Note: *Local alignment is not recommended for 2.1M, 3x720K, or 12x135K 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. Analyze Data

1. To start a copy number analysis, select **Analysis -> CGH-DNACopy** or **Analysis -> CGH-segMNT**.

   Note: *Roche NimbleGen recommends analyzing your data with the segMNT algorithm, described below, which exhibits increased accuracy and performance when compared with the DNACopy algorithm. Refer to the NimbleScan v2.5 User's Guide for detailed instructions for DNACopy analysis.*

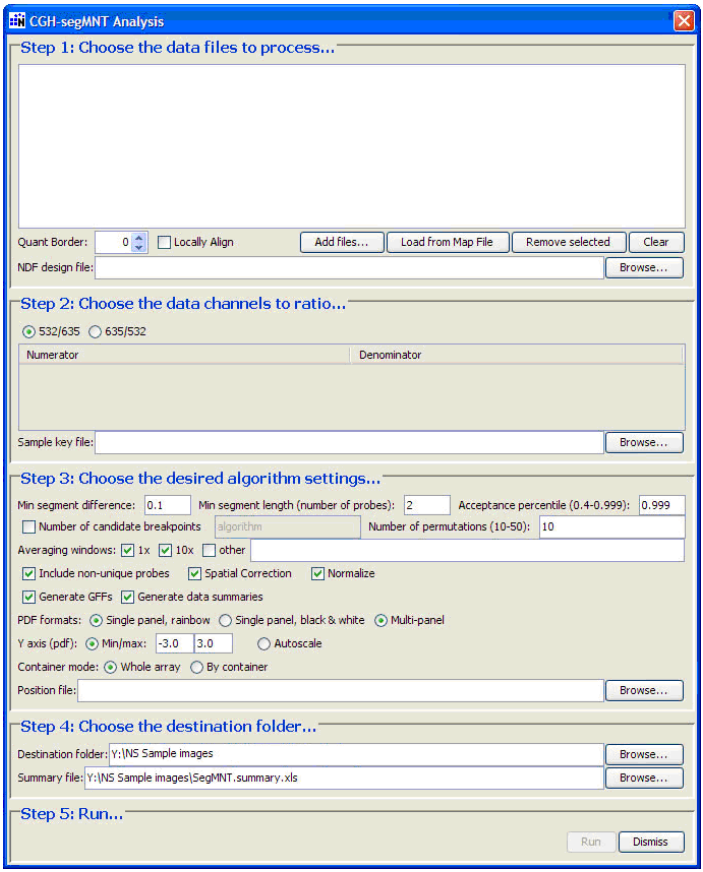


Figure 22: CGH-segMNT Analysis Dialog Box

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 or the gridded .tif files generated in Step 3 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**.

Note: *All .pair or .tif files selected must be from arrays that contain the same design.*

- *Quant Border.* (Specify if processing gridded .tif files) The quantification border dropdown menu offers a means to fine-tune feature quantification by decreasing or increasing the pixels to sample. By default, this value is set to zero (0).
- *Locally Align.* (Specify if processing gridded .tif files) Click the *Locally Align* checkbox to shift each quantification square for a feature (without overlapping the adjacent features) to find the maximum intensity for that feature. By default, this setting is not applied.

  Note: *Local alignment should not be used with 2.1M, 3x720K, or 12x135K arrays.*
- *NDF design file.* (Specify if processing gridded .tif files) The design (.ndf) file describes the placement of your probes on the microarray. Refer to Appendix A. File Descriptions and Examples in the *NimbleScan User's Guide* for an example of a design file.

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

- *532/635* or *635/532*. These option buttons allow you to select the data channels to ratio. By default, the 532/635 option button is selected.
- *Sample key file*. Select the sample key file, which maps data file names to samples and eliminates mistakes when processing a large number of samples. Following is an example of a sample key file.

```
ARRAY_ID  CY3_SAMPLE_NAME  CY5_SAMPLE_NAME
25748     TEST SAMPLE 1    CONTROL gDNA
25957     TEST SAMPLE 2    CONTROL gDNA
```

Figure 23: Example of a SampleKey.txt File

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

- *Min segment difference.* This value represents the minimum difference in the $\log_2$ ratio that two segments must exhibit before they are identified as separate segments. By default, the minimum value is set to 0.1. Increasing this value reduces the number of segments with smaller $\log_2$-ratio shifts.
- *Min segment length (number of probes).* This value represents the minimum number of consecutive probes that must exhibit a change in $\log_2$ ratio in order to call a segment. By default, this value is set to two probes. Increasing this value reduces the number of shorter segments.

- *Acceptance percentile (0.4 - 0.999).* This value represents the stringency with which initial segment boundaries are selected. By default, this value is set to 0.999. Higher values are more stringent, meaning that segment breakpoints are less likely to start or end at subtly-shifted data points.
- *Number of candidate breakpoints.* This value represents the number of data points to be analyzed to determine whether a segment should start or end there. By default, this value is set by the algorithm according to number of data points in the chromosome to be segmented. The number of candidate breakpoints can be adjusted and impacts the algorithm's sensitivity: too few and not all of the breakpoints will be found, too many and the analysis time increases greatly.
- *Number of permutations (10 - 50).* This value represents the number of times segmentation is performed on simulated pure noise data sets for the purpose of distinguishing significant segments from insignificant segments. By default, this value is set to 10. The segMNT algorithm uses permutation to justify the number of segments to output as the results.
- *Averaging window*s. Choose the window size to use when the software performs a set of transformations on the raw signal data of all probes within that window and reduces the raw data to a single data point. For example, when a 10x averaging window is applied to a whole-genome tiling-path array in which probes are spaced every 1,000bp across the genome, each window-averaged datapoint represents the average of approximately 10 original data points contained within a 10,000bp window. By default, the software uses 1x (unaveraged) and 10x averaging windows. You may specify other averaging windows in the dialog box (e.g. 5x, 20x, 50x, etc.). Note that when averaging windows, noise may be reduced at the expense of resolution.

  Note: *Averaging windows may be specified as described above (e.g. 5x, 20x) or by indicating the number of bp contained within each window (e.g. 5,000, 20,000). If the averaging window is smaller than the spacing between probes, the data is not averaged.*
- *Include non-unique probes.* Choose whether to include non-unique probes in segmentation analysis. In most cases, only unique probes (frequency = 1) are included in the array design. The design file includes the frequency count of each probe sequence within the genome. However, some designs include genomic regions that are duplicated in the genome (e.g. segmental duplications) and may thus contain non-unique probes (frequency > 1). By default, the *Use non-unique probes?* checkbox is selected. To omit non-unique probes from analysis, deselect this checkbox.

Note: *Unique probes are always included in segmentation analysis regardless of the inclusion or exclusion of non-unique probes.*

- *Spatial Correction.* Provides an option for applying a spatial correction to the raw data. Roche NimbleGen has found that spatial correction reduces some artifacts observed in CGH data from 2.1M arrays and has minimal impact on overall noise and $\log_2$-ratio values in regions of copy number variation. When spatial correction is applied, it corrects position-dependent non-uniformity of signals across the array. Specifically, locally weighted polynomial regression (LOESS) is used to adjust signal intensities based on X,Y feature position. By default, the checkbox is selected to enable spatial correction. When enabled, spatial correction is applied to the raw data signal intensities of each feature before normalization and segmentation analysis.
- *Normalize.* Choose whether to apply qspline fit normalization (Workman, et al. 2002) to the data prior to segmentation analysis. By default, normalization is applied. Normalization compensates for inherent differences in signal between the two dyes. However, it may not be desirable for all data sets including, for example custom fine-tiling designs with large regions of copy number changes.
- *Generate GFFs.* Choose whether to generate GFF (General Feature Format, .gff) files with $\log_2$-ratio data of the two channels. The software generates GFF files by default. These files offer a simple and interactive means to view and analyze CGH data.
- *Generate data summaries.* Choose whether to generate data summary files (.txt).
- *PDF formats.* Select the PDF format or formats in which to report CGH data. By default, NimbleScan software generates single panel rainbow and multi-panel plots.
    - *Single panel, rainbow plot.* Single panel, rainbow PDF plots show the $\log_2$ ratio for all probes plotted versus genomic position for all chromosomes or regions in a single plot with each chromosome or region differentiated by color and vertical dashed lines.
    - *Single panel, black & white plot.* Single panel, black & white PDF plots show the $\log_2$ ratio for all probes plotted versus genomic position for all chromosomes or regions in a single plot with each chromosome or region differentiated by vertical dashed lines.
    - *Multi-panel plot.* Multi-panel PDF plots show the $\log_2$-ratio data on a chromosome-by-chromosome (or region-by-region) basis.

- *Y axis (pdf)*. For single panel and multi-panel PDF plots, choose the Y axis (log_2-ratio) scale. Choose from the following options:
  - *Min/max*. Select this option to use a minimum/maximum range of values. Probes that exhibit log_2-ratio values below or above these values are excluded from the PDF plot. By default, this option is selected with a minimum Y axis of -3.0 and a maximum of 3.0. You may also specify other minimum and maximum Y axis values.
  - *Autoscale*. Select this option to generate PDF plots in which the Y axis is automatically set to encompass all the probes on the array.
- *Container mode*. Choose whether to combine or segregate probe sets based on the container in which they reside. Some NimbleGen array designs contain on-chip replicates scattered randomly across the array or separated into physically distinct blocks on the array. Each replicate is identified by its unique container name (usually enumerated as BLOCK1, BLOCK2, etc. or FORWARD1, FORWARD2, etc.). Replicate containers generally contain exact duplicates of the probe set and can be assessed independently (*By container* option) or aggregated (*Whole array* option). If your array does not contain on-chip replicates, these two options are equivalent.
- *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.

5. In *Step 4: Choose the destination folder...*:
   - *Destination folder*. Type the path to the folder or click **Browse** to open a dialog box for navigating to the location where analysis results will be stored.
   - *Summary file (.xls)*. Type the path to the folder or click **Browse** to open a dialog box for navigating to the location where the summary file will be stored and to designate the name of the summary file. This file provides metrics specific for CGH analysis (refer to "Reviewing the Summary File" on page 62 for a more detailed description of these metrics).
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 20 minutes to complete using the default window-average settings.

Step 8. Review Your Data

NimbleScan software can produce the following files for CGH data:

- Segmentation PDF plots (.pdf): Show CGH data in single panel rainbow plots or multi-panel plots.
- GFF files (general feature format, .gff): Contain the $\log_2$ ratio of Cy3 and Cy5 for each probe plotted versus genomic position.
- Data summary files (.txt): One set of these files contains the $\log_2$-ratio data of all data points. A second set contains a summary of predicted segments.

Below you learn how to review these files.

Note: *For detailed information on the file formats of PDF plots, GFF files, and data summary files, refer to the NimbleScan v2.5 User's Guide.*

Reviewing Segmentation PDF Plots

You review segmentation PDF plots (.pdf) using Adobe Acrobat Reader.

1. Open Acrobat Reader.
2. Select **File** -> **Open** and open .pdf plots generated by the CGH data analysis (hold down Ctrl and click to select multiple files). Be aware of the following when selecting .pdf plots:
    - NimbleScan software can generate .pdf plots in two formats:
        - Single panel rainbow plots show the $\log_2$ ratio for all probes plotted versus genomic position for all chromosomes or regions in a single plot with each chromosome or region differentiated by vertical dashed lines.
        - Multi-panel plots show the data on a chromosome-by-chromosome (or region-by-region) basis.
    - NimbleScan software can generate .pdf plots for unaveraged (1x) and window-averaged data. Roche NimbleGen recommends generating and viewing 10x window-averaged .pdf plots for an initial snapshot overview of your data.

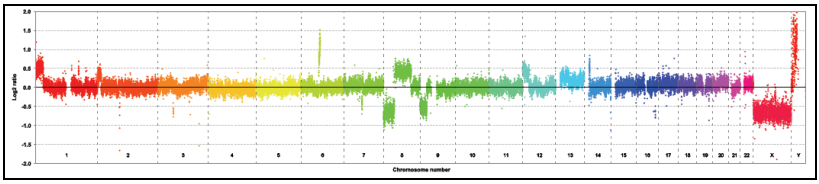


Figure 24: Human CGH Whole-Genome Array Data Displayed as a Single Panel Rainbow Plot

Reviewing GFF Files

You review GFF files (.gff) using SignalMap software. For more information on using SignalMap software, refer to the *SignalMap User's Guide* provided with the software.

1. Open SignalMap software.
2. Select **File** -> **New** and import .gff files generated by the CGH data analysis (hold down Ctrl and click to select multiple files). Be aware of the following when selecting .gff files:
   - NimbleScan software can generate .gff files containing normalized (no segmentation algorithm applied), unaveraged (1x), and window-averaged data.
   - Roche NimbleGen recommends generating and viewing the unaveraged .gff files for a comprehensive and highest-resolution view of your data.

   Tip: *You can drag and drop files into the SignalMap window to open.*
3. (Optional) Import the *design_ID*.gff file that contains gene annotation specific to your design.
   a. Insert the CD/DVD provided with your array(s), which includes the design_ID.gff file, into the appropriate drive.
   b. Select **File** -> **Import** and import the *design_ID*.gff file into SignalMap software.

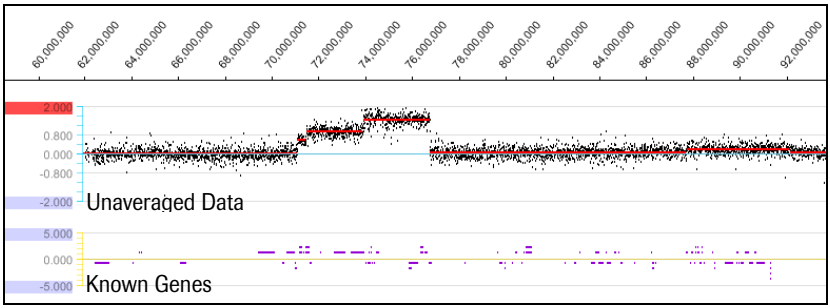


Figure 25: Array CGH Data Corresponding to a Region of Human Chromosome 6. A .gff annotation track showing known genes is displayed in parallel to the unaveraged (normalized and segmented) array CGH data in SignalMap software.

4. (Optional - for human hg18 designs only) Download and import the human hg18 annotation files. Appendix A provides detailed information about the human hg18 annotation files offered by Roche NimbleGen.

   a. In a web browser, download Roche NimbleGen hg18 annotation files from *www.nimblegen.com/human-annotation*.

   b. In SignalMap software, select **File** -> **Import** and import the human hg18 annotation files (hold down Ctrl and click to select multiple files).

5. Review your data using these SignalMap functions:

   - **Select chromosomes**: Use the pane selector field below the toolbar to change the view to either all chromosomes (All Tracks) or a selected chromosome (e.g. chr2).
   - **Set the log_2-ratio scale**: To compare multiple .gff tracks, set the log_2-ratio scale so it is the same for all selected tracks:

     a. Select **Edit** -> **Select All** (hold down the Ctrl key and click the Y axis to select individual tracks).

     b. Select **View** -> **Manual Scale**. Enter the desired minimum and maximum scale values (Roche NimbleGen recommends starting with a scale from -2 to 2) and click **OK**.

   - **Set track height**: Select the desired .gff tracks as described above, select **Track** -> **Set Height**, enter the desired track height (Roche NimbleGen recommends 120), and click **OK**.

Appendix B describes additional SignalMap functions you can use when reviewing .gff files.

Reviewing Data Summary Files

You review data summary files (.txt) using spreadsheet software (e.g. Microsoft Excel) or a text editor (e.g. Microsoft WordPad).

1. Open spreadsheet software or a text editor.

   Tip: *Spreadsheet software allows you to sort segments by genomic position, size, or log_2-ratio mean.*

2. Select **File** -> **Open** and open data summary files (.txt) generated by the CGH data analysis (hold down Ctrl and click to select multiple files). Be aware of the following when selecting data summary files:

   - NimbleScan software can generate one set of data summary files that lists log_2-ratio values for all data points. These .txt files can be generated for non-segmented data (.txt), unaveraged (1x) data (unavg.txt), and window-averaged data (avg.txt).
   - NimbleScan software can generate a second set of data summary files that lists the predicted segments and their corresponding genomic position (by chromosome and base pair coordinates), size, log_2 ratio, and number of data points contained within the segment. These .txt files can be generated for unaveraged (1x) data (unavg_segtable.txt) and window-averaged data (segtable.txt).

Reviewing the Summary File

You review the summary file (.xls) using spreadsheet software (e.g. Microsoft Excel) or a text editor (e.g. Microsoft WordPad).

1. Open spreadsheet software or a text editor.
2. Select **File** -> **Open** and open the summary file (.xls) generated by the CGH data analysis.

IMAGE_ID	mad.1dr	chrX
1950702_01_532	0.208	0.028591179
1950702_01_635	0.208	0.028591179

Figure 26: Example of a Summary File

Column Header	Description
IMAGE_ID	Name of the image file (minus the .tif extension) the data was extracted from. For NimbleGen image files, this is the array ID, followed by an underscore, plus additional information, such as the wavelength used to scan the array.
mad.1dr	The median absolute deviation of the $\log_2$-ratio difference between consecutive probes. This metric provides a surrogate measure of experimental noise.
CHR-X	The mean $\log_2$-ratio of the largest segment detected in Chromosome X from experiments using sex-mismatched samples.

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 1.8.	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 1.8.	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 genomic DNA extraction.
Gel image shows a smear of DNA and/or large amounts of low molecular weight material.	DNA sample is contaminated with RNA.	Perform RNase A digestion followed by phenol/ chloroform extraction and ethanol precipitation.
	DNA sample is degraded.	Repeat genomic DNA extraction.

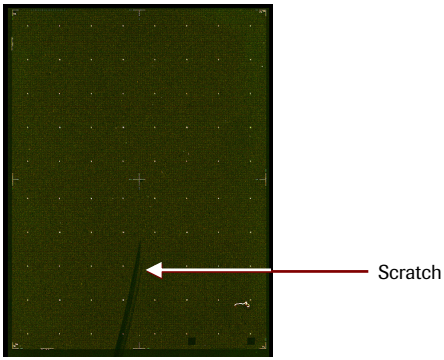
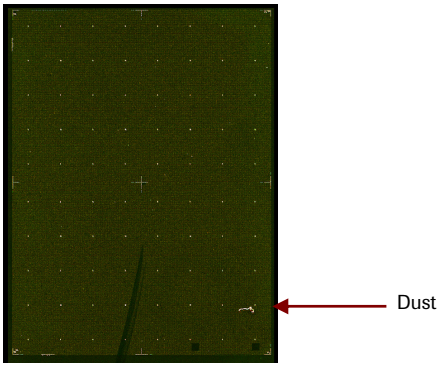
Labeling

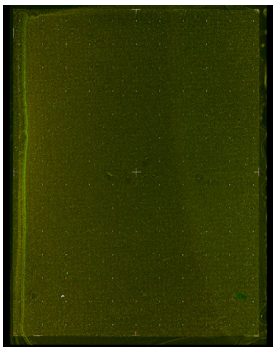
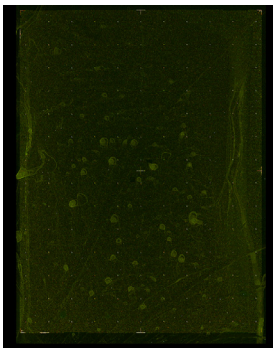
Problem	Possible Cause	Recommended Corrective Action
Labeling yield is less than 10µg per reaction (or less than 34µg total for 2.1M arrays).	DNA sample is contaminated or degraded.	Check genomic DNA absorption ratios and gel image. If necessary, clean up samples or repeat genomic DNA extraction. Repeat labeling.
	Random Primer Buffer is missing ß-Mercaptoethanol or diluted primers, or is older than 4 months. Primers were not diluted correctly.	Check the age of the diluted Random Primer Buffer and that the ß-Mercaptoethanol was added. If necessary, prepare a fresh dilution of nonamer primers. 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

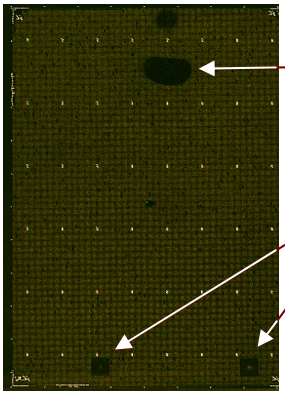
Problem	Possible Cause	Recommended Corrective Action
Mixer is poorly aligned on slide.	The slide was not flush in the PMAT.	Remove the mixer using the Mixer Disassembly Tool then reassemble using a new mixer.
	PMAT is not properly calibrated (multiple mixers misalign).	Send PMAT to Roche NimbleGen for calibration.
Hybridization solution does not enter the hybridization chamber.	The pipette tip is not situated properly on the fill port.	Refer to page 27 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 27 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 25 for proper braying technique. Repeat hybridization.
	The mixer port seals/multi-port seals were not fully adhered to the mixer.	Refer to page 28 for proper sealing technique. Ensure that excess hybridization solution has been wiped from the ports before adhering the mixer port seal/ multi-port seal. Repeat hybridization.

Scanning

Problem	Possible Cause	Recommended Corrective Action
Scratches and/or fingerprints are seen on the array image. 	The slide was mishandled or dropped.	Grip the slide only on its edges and handle with care.
Dust is seen on the array image. 	The array was exposed to environmental dust.	If the amount of dust present is small, use the NimbleGen Compressed Gas Nozzle to gently blow compressed inert gas across the array to remove the dust. Rescan the array. If the amount of dust is excessive, repeat wash, 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 wash, 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.
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 1,400rpm 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 36 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.	PMT settings are not adjusted correctly.	Refer to page 36 for instructions on how to adjust PMT settings. Repeat scan.
	Hybridization conditions are too stringent.	Check that the hybridization solution was prepared correctly (refer to page 22) and the NimbleGen Hybridization System is set to and maintaining 42°C. Repeat hybridization.
	Sample leaked out of the mixer during hybridization due to incomplete braying.	Refer to page 25 for proper braying technique. Repeat hybridization.

Problem	Possible Cause	Recommended Corrective Action
The array image appears dim. *(continued)*	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 20ppb and humidity levels below 40%. Repeat hybridization.
The fiducial features appear dim or blank.	The alignment oligo was either not added to the hybridization solution or was degraded due to repeated freeze-thaw cycles.	Repeat hybridization, using fresh alignment oligo, if necessary.
Features appear blank on portions of the array.	The slide contains a scratch or fingerprint.	Grip the slides only on the edges and handle with care. 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.



Problem	Possible Cause	Recommended Corrective Action
The array image is too bright.	Hybridization conditions are not stringent enough.	Check that the hybridization solution was prepared correctly (refer to page 22) and the NimbleGen Hybridization System power is on, mixing is on, and temperature is set to and maintaining 42°C. Repeat hybridization.
	PMT settings are not adjusted correctly.	Refer to page 36 for instructions on how to adjust PMT settings. Repeat scan.
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 25 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 30 for proper washing technique. Repeat hybridization.
The array image is uniformly yellow and lacks red or green saturated features.	The hybridization conditions are not stringent enough.	Check that the hybridization solution was prepared correctly (refer to page 22) and the NimbleGen Hybridization System power is on, mixing is on, and temperature is set to and maintaining 42°C. Repeat hybridization.

Problem	Possible Cause	Recommended Corrective Action
The array image is either too green or too red.	The PMT settings are not adjusted correctly.	Refer to page 36 for instructions on how to adjust the PMT settings. Repeat scan.
	Test and reference 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.	Scanner is out of alignment.	Refer to the scanner user's guide. Standard procedure is to grid each image (Cy3 and Cy5) individually, then generate pair reports so that data quality is not compromised. Scanner calibration/service is recommended.

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.	Test and reference 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 25 for proper braying technique. ■ Excess sample is removed from around the loading ports. Refer to page 28 for proper sealing technique. ■ The mixer port seals/multi-port seals were not fully adhered to the mixer after loading sample. Refer to page 28 for proper sealing technique.

Data Analysis

Problem	Possible Cause	Recommended Corrective Action
Data appear noisy.	DNA sample was of poor quality.	Refer to Chapter 2. Sample Preparation & QC (page 11). Repeat labeling and hybridization.
	Species/strain variation between test and reference samples.	If test and reference samples are of different species or strains, data may be noisy.
Data show periodic waves.	DNA sample may be of poor quality.	Refer to Chapter 2. Sample Preparation & QC (page 11). Repeat labeling and hybridization.
No copy number changes are detected.	Hybridization conditions are not stringent enough.	Check that the hybridization solution was prepared correctly (refer to page 22) and the NimbleGen Hybridization System power is on, mixing is on, and temperature is set to and maintaining 42°C. Repeat hybridization.
The log_2-ratio values are muted.	Samples are not 100% pure (e.g. mosaicism).	Mosaic samples will yield lower than expected log_2-ratio values.
	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.
	DNA sample was of poor quality.	Refer to Chapter 2. Sample Preparation & QC (page 11). Repeat labeling and hybridization.

Problem	Possible Cause	Recommended Corrective Action
The log_2-ratio values are muted. *(continued)*	Hybridization conditions are not stringent enough.	Check that the hybridization solution was prepared correctly (refer to page 22) and the NimbleGen Hybridization System power is on, mixing is on, and temperature set to and maintaining 42°C. Repeat hybridization.
NimbleScan fails to open the image TIFF file or Multiplex images fail to burst.	The wrong .ncd file was specified.	Refer to page 41 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 36 for instructions on how to specify the scan area. Check the image in GenePix software and rescan.
	The scanned area is too large.	Refer to page 36 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 36 for instructions on how to specify the scan area. Crop the image in NimbleScan software or rescan if necessary.

Problem	Possible Cause	Recommended Corrective Action
The auto align function fails to grid the array image.	The fiducial features are dim.	Perform a manual alignment as described in the *NimbleScan User's Guide*.
	NimbleScan v2.4 or higher 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 User's Guide*.

Appendix A. Annotation Files Available for Human HG18 Array Designs

Roche NimbleGen offers a suite of annotation .gff files based on build hg18 of the human genome. You can download these files at *www.nimblegen.com/human-annotation*, and then import and view them alongside your experimental data in SignalMap software. Each annotation .gff track includes Internet links, allowing you to click on a particular annotation feature and access its entry in the relevant online database.

- **Genes.gff track**: Indicates all genes for build hg18 as reported in the UCSC Genome browser (***http://genome.ucsc.edu***). Genes annotated above the baseline in each track represent features identified on the sense strand, while entries below the baseline represent features identified on the antisense strand.
- **Genes_Exon-Intron.gff track**: Indicates the exon-intron boundaries of all genes in build hg18 as reported in the UCSC Genome browser. Exons are denoted as dark blue bars, and introns are denoted as light blue bars.
- **Transcription_Start_Sites .gff track**: Indicates all transcription initiation sites for build hg18 as reported in the UCSC Genome browser.

  Note: *Features in this track may be more easily viewed by changing the feature style from dots to bars. To do this, select the track by clicking its Y axis and select* ***Track*** *->* ***Style*** *->* ***Bars****.*
- **Structural_Variants.gff track**: Displays all copy number variants as reported in the Database of Genomic Variants (***http://projects.tcag.ca/variation***).
- **Segmental_Duplications.gff track**: Displays regions of genomic duplication >1kb in size and with >90% sequence identity after masking high-copy repeat regions (Bailey, et al. 2001; 11:1005-17) and reported in the UCSC Genome browser. The level of similarity is indicated as follows: light to dark gray bars = 90 - 98% similarity, light to dark yellow bars = 98 - 99% similarity, light to dark orange bars ≥99% similarity; red = duplications of >98% that lack sufficient evidence in the Segmental Duplication database.

- **Cytogenetic_Ideogram.gff track**: Displays the cytogenetic bands, in grayscale format, for each chromosome as reported in the UCSC Genome browser.
- **miRNA.gff track**: Indicates all miRNAs as reported in the miRBase database (*http://microrna.sanger.ac.uk/*). Each feature represents the entire hairpin sequence for the miRNA.

  Note: *Features in this track may be more easily viewed by changing the feature style from dots to bars. To do this, select the track by clicking its Y axis and select* ***Track*** *->* ***Style*** *->* ***Bars****.*

Appendix B. Additional Techniques for Reviewing GFF Files

The following SignalMap functions are also helpful when reviewing .gff files:

- **Zoom**: Select the magnifier button on the toolbar. Position the magnifier cursor to the region of interest then click and drag to draw a bounding box around the region to magnify. Alternatively, hold down the Ctrl key and press + to zoom in or - to zoom out.
- **Arrange tracks**: To move a data track above or below another track, click in the left margin of the track, move the cursor until a gray dashed line appears, and then click to place the track in the new position.
- **Search for genes**: To search for a particular gene by gene name or accession number, select **Edit** -> **Search**. Type the gene name or accession number in the search field and click **Find**. To jump to the data track where the gene is located, click **Go to Selected**.
- **Pointer information**: To gather information about a specific data segment or annotation feature, click the pointer button on the toolbar and position the cursor over the region of interest. For data segments, the $\log_2$ ratio and genomic position will be displayed in the top left corner of the SignalMap window. For annotation features, details including gene name, cytoband coordinates, and CNV reference information from the Database of Genomic Variants will be displayed.
- **Attach Cursor**: To move quickly from one data point or annotation feature to the next, click the Y axis of the track of interest and select **Cursor** -> **Attach Cursor**. A vertical line will appear at the left-most feature of the track. To jump to the next feature, use the left and right arrows. To remove the cursor from the track, select **Cursor** -> **Detach Cursor**. This function is particularly useful when there are large gaps between data points or annotation features.

Notes

Appendix C. Limited Warranty

ROCHE NIMBLEGEN, INC.
NIMBLEGEN ARRAYS

1. Limited Warranty

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Notes

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