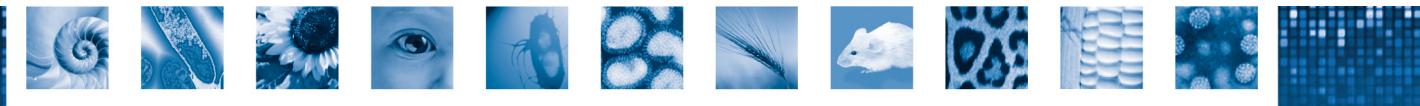




# NimbleGen Array User's Guide

## *Gene Expression Arrays*

Version 5.0



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



---

## Copyright

© 2010 Roche NimbleGen, Inc. All Rights Reserved.

## Edition

Version 1.0, 16 Jan 2007; Version 2.0, 27 Aug 2007; Version 3.0, 21 May 2008; Version 3.1, 24 Oct 2008; Version 3.2, 11 Mar 2009;  
Version 4.0, 31 Dec 2009; Version 5.0, 28 May 2010

## Service Marks and Trademarks

NIMBLEGEN is a trademark of Roche.

Other brands, product names, company names, trademarks, and service marks are the properties of their respective holders.

## Restrictions and Liabilities

This document is provided "as is" and Roche NimbleGen, Inc. ("Roche NimbleGen") assumes no responsibility for any typographical, technical, or other inaccuracies in this document. Roche NimbleGen reserves the right to periodically change information that is contained in this document; however, Roche NimbleGen makes no commitment to provide any such changes, updates, enhancements, or other additions to this document to you in a timely manner or at all.

OTHER THAN THE LIMITED WARRANTY CONTAINED IN THIS USER GUIDE, ROCHE NIMBLEGEN MAKES NO REPRESENTATIONS, WARRANTIES, CONDITIONS OR COVENANTS, EITHER EXPRESS OR IMPLIED (INCLUDING WITHOUT LIMITATION, ANY EXPRESS OR IMPLIED WARRANTIES OR CONDITIONS OF FITNESS FOR A PARTICULAR PURPOSE, NON-INFRINGEMENT, MERCHANTABILITY, DURABILITY, TITLE, OR RELATED TO THE PERFORMANCE OR NON-PERFORMANCE OF ANY PRODUCT REFERENCED HEREIN OR PERFORMANCE OF ANY SERVICES REFERENCED HEREIN).

This document might contain references to third party sources of information, hardware or software, products, or services and/or third party web sites (collectively the "Third-Party Information"). Roche NimbleGen does not control, and is not responsible for, any Third-Party Information, including, without limitation the content, accuracy, copyright compliance, compatibility, performance, trustworthiness, legality, decency, links, or any other aspect of Third-Party Information. The inclusion of Third-Party Information in this document does not imply endorsement by Roche NimbleGen of the Third-Party Information or the third party in any way.

Roche NimbleGen does not in any way guarantee or represent that you will obtain satisfactory results from using NimbleGen arrays as described herein. The only warranties provided to you are included in the Limited Warranty enclosed with this guide. You assume all risk in connection with your use of NimbleGen arrays.

Roche NimbleGen is not responsible nor will be liable in any way for your use of any software or equipment that is not supplied by Roche NimbleGen in connection with your use of NimbleGen arrays.

## Conditions of Use

NimbleGen arrays are intended for life science research only and are not for use in diagnostic procedures. You are responsible for understanding and performing the protocols described within. Roche NimbleGen does not guarantee any results you may achieve. These protocols are provided as Roche NimbleGen's recommendations based on its use and experience with NimbleGen arrays.

Roche NimbleGen does not guarantee that any NimbleGen array may be used for multiple hybridizations nor does it take any responsibility for your successful satisfaction of the NimbleGen reuse protocol. Reuse of NimbleGen arrays is at your own risk and liability.

## Use Restrictions

THIS PRODUCT, AND/OR COMPONENTS OF THIS PRODUCT, ARE LICENSED BY AFFYMETRIX UNDER CERTAIN PATENTS OWNED BY AFFYMETRIX. THIS PRODUCT IS LICENSED FOR LIFE SCIENCE RESEARCH ONLY AND IS NOT FOR USE IN DIAGNOSTIC PROCEDURES. THIS LIMITED LICENSE PERMITS ONLY THE USE OF THIS PRODUCT FOR RESEARCH PURPOSES. NO OTHER RIGHT, EXPRESS OR IMPLIED, IS CONVEYED BY THE SALE OF THIS PRODUCT. THE PURCHASE OF THIS PRODUCT DOES NOT BY ITSELF CONVEY OR IMPLY THE RIGHT TO USE SUCH PRODUCT IN COMBINATION WITH ANY OTHER PRODUCT(S) WHOSE MANUFACTURE, SALE OR USE IS COVERED BY AN AFFYMETRIX PATENT. NO RIGHT TO MAKE, HAVE MADE, USE, IMPORT, OFFER TO SELL, OR SELL ANY OTHER PRODUCT IN WHICH AFFYMETRIX HAS PATENT RIGHTS (INCLUDING WITHOUT LIMITATION MICROARRAY READERS OR DETECTORS OR SOFTWARE FOR USE WITH MICROARRAY READERS OR DETECTORS) IS IMPLIED BY THE SALE OR PURCHASE OF THIS PRODUCT.

# Table of Contents

<b>Chapter 1. Before You Begin .....</b>	<b>5</b>
What's New? .....	5
Components Supplied.....	7
Microarray Storage .....	7
Protocol Information & Safety .....	7
Required Equipment, Labware & Consumables.....	8
NimbleGen Equipment.....	8
Software .....	8
Standard Laboratory Equipment.....	9
Consumables & Accessories Available from Roche NimbleGen .....	9
Reagents/Consumables Purchased from Other Vendors.....	11
Technical Support .....	12
Conventions Used in This Manual.....	12
<b>Chapter 2. Sample Preparation &amp; QC .....</b>	<b>13</b>
Sample Requirements.....	13
Step 1. Spectrophotometric QC of RNA .....	13
Step 2. Bioanalyzer/Gel QC of RNA .....	13
Step 3. First Strand cDNA Synthesis.....	15
Step 4. Second Strand cDNA Synthesis.....	16
Step 5. RNase A Cleanup.....	16
Step 6. cDNA Precipitation.....	17
Step 7. Spectrophotometric QC of cDNA.....	17
Step 8. Bioanalyzer/Gel QC of cDNA.....	17
<b>Chapter 3. Sample Labeling.....</b>	<b>19</b>
<b>Chapter 4. Hybridization &amp; Washing .....</b>	<b>23</b>
Step 1. Prepare Samples.....	23
Step 2. Prepare Mixers.....	24
Step 3. Load & Hybridize Samples.....	26
Step 4. Wash Hybridized Arrays.....	29
<b>Chapter 5. One-Color Array Scanning.....</b>	<b>33</b>
Step 1. Start Control Unit, Turn on Scanner & Load Slides .....	33
Step 2. Start the Software & Turn on the Lasers .....	34
Step 3. Set Scan Parameters Using the Software .....	35
Step 4. Scan the Slides.....	38
Step 5. Review Scanned Images .....	40
<b>Chapter 6. NimbleScan Data Analysis.....</b>	<b>41</b>
Step 1. Burst Multiplex Image (4x72K & 12x135K Arrays Only) .....	41
Step 2. Import Image.....	41
Step 3. Extract Image.....	42
Step 4. Confirm Experimental Integrity (4x72K & 12x135K Arrays Only) .....	42
Performing a Sample Tracking Analysis .....	44
Visually Checking STC Features.....	46

*Table of Contents*

---

Step 5. Generate an Experimental Metrics Report .....	46
Step 6. (Optional) Create Pair Reports .....	50
Step 7. Analyze Data.....	50
<b>Chapter 7. Troubleshooting .....</b>	<b>53</b>
Sample Quality.....	53
Labeling.....	53
Hybridization.....	54
Scanning.....	55
Sample Tracking Controls (STCs).....	59
Data Analysis.....	60
<b>Appendix A. Expression Data Analysis.....</b>	<b>61</b>
Step 1. Install the Necessary Software.....	61
Step 2. Import Your Expression Data.....	61
Step 3. Analyze Your Expression Data .....	61
<b>Appendix B. Limited Warranty.....</b>	<b>65</b>

# Chapter 1. Before You Begin

This *User's Guide* describes the processes for analyzing gene expression using these NimbleGen array formats:

- 385K (385,000 features)
- 4x72K (4 x 72,000 features)
- 12x135K (12 x 135,000 features)

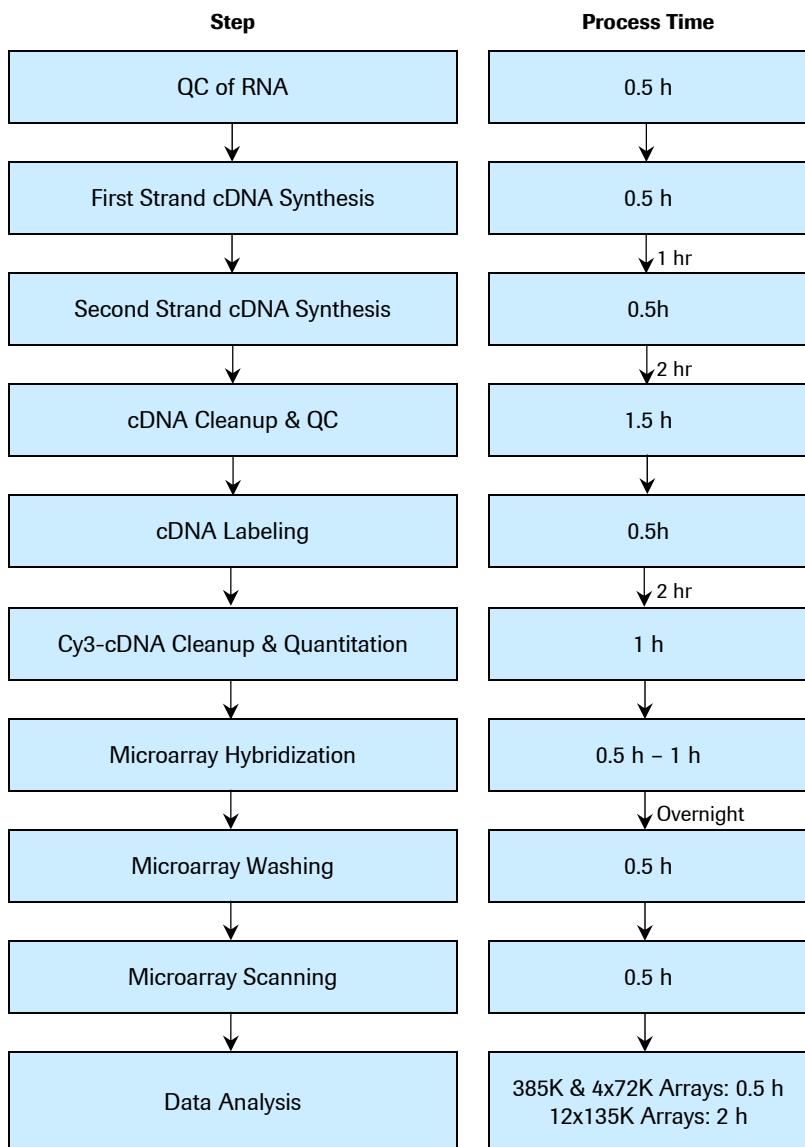
The first step involves creating cDNA from eukaryotic total RNA or poly-A<sup>+</sup> RNA (mRNA) and cDNA from prokaryotic total RNA using the Invitrogen SuperScript Double-Stranded cDNA Synthesis Kit. The second step requires labeling the cDNA using a NimbleGen One-Color DNA Labeling Kit and subsequent hybridization to a NimbleGen array using a NimbleGen Hybridization System. Subsequent steps include washing, drying, and scanning the array. Finally, data is extracted from the scanned image and analyzed for gene expression levels.

## What's New?

Version 5.0 of this *User's Guide* includes instructions on using the redesigned HX12 mixer and NimbleGen v2.6 Software for gene expression experiments.



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



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

## Components Supplied

Component	Description
NimbleGen Arrays	As ordered
NimbleGen Mixers	<ul style="list-style-type: none"> <li>■ One X1 mixer is <i>ordered separately</i> from a 385K array.</li> <li>■ One X4 mixer is provided per 4x72K array.</li> <li>■ One HX12 mixer is provided per 12x135K array.</li> </ul>
Mixer Port Seals or Mixer Multi-port Seals	<p>For sealing fill and vent ports of NimbleGen mixers:</p> <ul style="list-style-type: none"> <li>■ Mixer ports seals are provided with X1 and HX12 mixers</li> <li>■ Mixer multi-port seals are provided with X4 mixers</li> </ul>
CD/DVD	This <i>User's Guide</i> and NimbleGen design files are included on the Design Information CD/DVD.

## Microarray Storage

NimbleGen arrays are packaged with desiccant and can be stored at room temperature for use by the expiration date. Once the seal is broken, store NimbleGen arrays in a desiccator at room temperature until use.

## Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- To avoid RNA degradation, Roche NimbleGen recommends using RNaseZap to eliminate RNase contamination from work surfaces.
- Keep RNA on ice at all times.
- Keep RNase A in a designated work area and use designated pipettes and tips to prevent RNase A contamination throughout the lab. Change gloves after handling RNase A and clean work area with RNaseZap immediately after use.
- Phenol:chloroform:isoamyl alcohol is dangerous and should be handled with caution. Wear the correct personal protective equipment when handling this chemical. Refer to MSDS for more information.
- Cy dyes are light sensitive. Be sure to minimize light exposure of the dyes during use and store in the dark when not in use.
- Cy dyes are ozone sensitive. Take the necessary precautions to keep atmospheric ozone levels below 20 ppb (parts per billion).
- Cy dyes are humidity sensitive. Take the necessary precautions to keep humidity levels below 40%.
- Roche NimbleGen has found that using VWR water and DTT for all post-hybridization washes results in higher signal from Cy dyes.



Reconstitute the DTT provided in the NimbleGen Wash Buffer Kit in a fume hood. [Chapter 4. Hybridization and Washing](#) provides details on how to reconstitute the DTT.

- Roche NimbleGen recommends using a NanoDrop Spectrophotometer for quantifying and characterizing nucleic acid samples because this instrument requires only 1.5 µl of sample for analysis.
- Perform all centrifugations at room temperature unless indicated otherwise.

## Required Equipment, Labware & Consumables

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

### NimbleGen Equipment

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

Equipment	Supplier	Process Quantity	Catalog No.
NimbleGen Hybridization System 4*	Roche NimbleGen	4 slides	05 223 652 001 (110V) 05 223 679 001 (220V)
NimbleGen Hybridization System 12*	Roche NimbleGen	12 slides	05 223 687 001 (110V) 05 223 695 001 (220V)
NimbleGen Microarray Dryer	Roche NimbleGen	24 slides	05 223 636 001 (110V) 05 223 644 001 (220V)
NimbleGen MS 200 Microarray Scanner	Roche NimbleGen	48 slides	05 394 341 001

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

### Software

Program	Supplier	Catalog No.
NimbleScan v2.6	Roche NimbleGen	05 933 315 001 (Individual License) 05 933 331 001 (Site License)

## Standard Laboratory Equipment

Equipment	Supplier	Catalog No.
Compressed Gas Nozzle	TeqCom	TA-N2-2000
Bioanalyzer	Agilent	2100
DNA Vacuum Concentrator	Thermo Savant	
Desiccator	<i>Multiple Vendors</i>	
Heat Block (capable of temperatures to 98°C)	<i>Multiple Vendors</i>	
Microcentrifuge (12,000 x g capability)	<i>Multiple Vendors</i>	
Microman M10 Pipette (recommended for 4x72K and 12x135K arrays)	Gilson	F148501
Microman M100 Pipette (recommended for 385K arrays)	Gilson	F148504
Spectrophotometer	NanoDrop	ND-1000 or newer
Thermocycler	<i>Multiple Vendors</i>	
Vortex Mixer	<i>Multiple Vendors</i>	

## Consumables & Accessories Available from Roche NimbleGen

Component	Package Size / Process Quantity	Catalog No.
NimbleGen X1 Mixer (for 385K arrays; includes mixer port seals)	5 mixers 10 mixers	05 391 717 001 05 223 725 001
NimbleGen X4 Mixer (for 4x72K arrays; includes mixer multi-port seals)	5 mixers 10 mixers	05 391 725 001 05 223 733 001
NimbleGen HX12 Mixer (for 12x135K arrays; includes mixer port seals)	5 mixers 10 mixers	05 391 768 001 05 223 768 001

Component	Package Size / Process Quantity	Catalog No.
NimbleGen One-Color DNA Labeling Kit	20 Cy3 labeling reactions Contents: <ul style="list-style-type: none"><li>■ Nuclease-free Water (2 x vial 1)</li><li>■ Random Primer Buffer (vial 2)</li><li>■ Cy3-Random Nonamers (vial 3)</li><li>■ Klenow Fragment (3'-&gt;5' exo-) 50 U/μl (vial 4)</li><li>■ 10mM dNTP Mix (vial 5)</li><li>■ Stop Solution (0.5 M EDTA) (vial 6)</li><li>■ 5 M NaCl (vial 7)</li></ul>	05 223 555 001
NimbleGen Hybridization Kit	100 hybridizations using 385K arrays Contents: <ul style="list-style-type: none"><li>■ 2X Hybridization Buffer (vial 1)</li><li>■ Hybridization Component A (vial 2)</li><li>■ Alignment Oligo<sup>1</sup> (vial 3)</li></ul>	05 583 683 001
NimbleGen Hybridization Kit, LS (Large Scale)	160 hybridizations using 4x72K arrays 156 hybridizations using 12x135K arrays Contents: <ul style="list-style-type: none"><li>■ 2X Hybridization Buffer (3 x vial 1)</li><li>■ Hybridization Component A (3 x vial 2)</li><li>■ Alignment Oligo<sup>1</sup> (3 x vial 3)</li></ul>	05 583 934 001
NimbleGen Sample Tracking Control Kit	480 hybridizations using 4x72K or 12x135K arrays Contents: Sample Tracking Controls <sup>2</sup>	05 223 512 001

Component	Package Size / Process Quantity	Catalog No.
NimbleGen Wash Buffer Kit	20 washes (processing up to 12 slides per wash)	05 584 507 001
Contents:		
■ 10X Wash Buffer I (2 x vial 1)		
■ 10X Wash Buffer II (vial 2)		
■ 10X Wash Buffer III (vial 3)		
■ DTT (2 x vial 4)		
■ Nuclease-free Water (3 x vial 5)		
NimbleGen Array Processing Accessories		05 223 539 001
Contents:		
■ Slide Rack		
■ Wash Tanks		
■ Slide Containers		
1 The Alignment Oligo is a mixture of Cy3 and Cy5 labeled 48 mer oligonucleotides that hybridize to alignment features on NimbleGen arrays. It is required for proper extraction of array data from the scanned image.		
2 Twelve Sample Tracking Controls (STCs) are provided. Each STC is a Cy3-labeled 48 mer oligonucleotide. When a unique STC is added to each sample before hybridization to a multiplex array, the STC can be used to confirm that the correct sample was hybridized to each array.		

### Reagents/Consumables Purchased from Other Vendors

Component	Supplier	Package Size	Catalog No.
β-Mercaptoethanol	Sigma Aldrich	25 ml	M3148
0.5 M EDTA	Sigma Aldrich	100 ml	E7889
5 mg/ml Glycogen	Ambion	5 x 1 ml	9510
7.5 M Ammonium Acetate	Sigma Aldrich	1 liter	A2706
Absolute Ethanol	Sigma Aldrich	500 ml	E702-3
Agilent RNA 6000 Nano Kit	Agilent	1 kit	5067-1511
Compressed Nitrogen or Argon Gas (for cleaning array surface)*	<i>Multiple Vendors</i>		
CP10 Pipette Tips (for 4x72K and 12x135K arrays)	Gilson	192 tips 960 tips	F148412 F148312
CP100 Pipette Tips (for 385K arrays)	Gilson	192 tips 960 tips	F148414 F148314
Isopropanol	Sigma Aldrich	500 ml	I-9516
Oligo dT Primer	Roche Applied Science	40 µg (1 A <sub>260</sub> unit, 8nmol)	10814270001
Phase Lock tubes (Light 1.5 ml)	Fisher Scientific	200 tubes	FP2302800
Phenol:chloroform: isoamyl alcohol (25:24:1)	Ambion	100 ml	9730

Component	Supplier	Package Size	Catalog No.
Random Hexamer Primer	Roche Applied Science	2 mg (50 A <sub>260</sub> units, 1 μmol)	11034731001
RNase A Solution	Promega	4 mg/ml	A7973
RNaseZap	Ambion	250 ml	AM9780
SuperScript	Invitrogen	10 reactions	11917-010
Double-Stranded cDNA Synthesis Kit		50 reactions	11917-020
Water: reagent grade, ACS, nonsterile, type 1	VWR	2.5 gallon	RC915025
Cotton Swabs	Multiple Vendors		

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

## Technical Support

If you have questions, contact your local Roche Microarray Technical Support. Go to [www.nimblegen.com/arraysupport](http://www.nimblegen.com/arraysupport) for contact information.

## Conventions Used in This Manual

### Text Conventions

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

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

### Symbols

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

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

# Chapter 2. Sample Preparation & QC

Chapter 2 describes how to create cDNA from eukaryotic total RNA or poly-A<sup>+</sup> RNA (mRNA), and cDNA from prokaryotic total RNA using the Invitrogen SuperScript Double-Stranded cDNA Synthesis Kit.

## Sample Requirements

- High-quality RNA is required for optimal cDNA synthesis yield and cDNA labeling for microarray hybridization.
- Roche NimbleGen recommends starting with the following amounts of total RNA or poly-A<sup>+</sup> RNA amounts for each hybridization:

Array Format	Eukaryotes	Prokaryotes
385K arrays	10 µg total RNA	10 µg total RNA
	1 µg poly-A <sup>+</sup> RNA	
4x72K arrays	10 µg total RNA	10 µg total RNA
	1 µg poly-A <sup>+</sup> RNA	
12x135K arrays	10 µg total RNA	10 µg total RNA
	1 µg poly-A <sup>+</sup> RNA	

## Step 1. Spectrophotometric QC of RNA

Prior to synthesizing cDNA, verify that the RNA samples are of sufficient purity.

1. Quantitate each RNA sample according to the following formula:

$$\text{RNA Concentration } (\mu\text{g/ml}) = A_{260} \times 40 \times \text{Dilution Factor}$$

RNA samples must have a concentration  $\geq 1.0 \mu\text{g}/\mu\text{l}$ .

2. Verify all samples meet the following requirements:

- $A_{260}/A_{280} \geq 1.8$
- $A_{260}/A_{230} \geq 1.8$

## Step 2. Bioanalyzer/Gel QC of RNA

Verify RNA samples are of sufficient molecular weight. Roche NimbleGen recommends the use of an Agilent 2100 Bioanalyzer because the required sample size is very small. If a Bioanalyzer is not available, use a denaturing agarose gel.

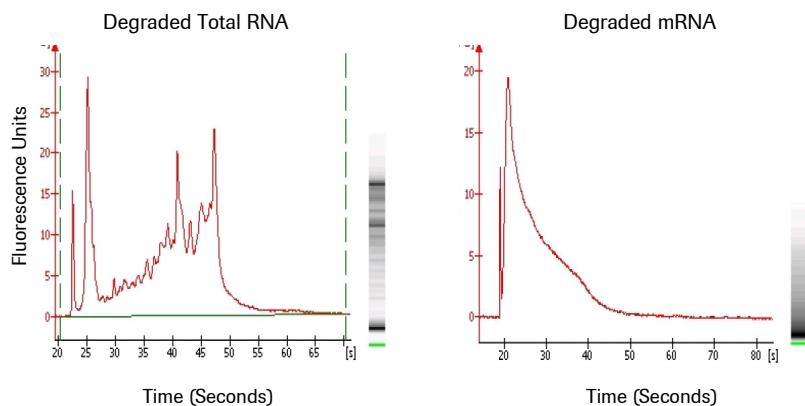
1. Transfer 250 ng total RNA or 250 ng poly-A<sup>+</sup> RNA to a sterile microcentrifuge tube. Store the remainder of your sample on ice or at -80°C.

2. Analyze samples using the Agilent Bioanalyzer and RNA 6000 Nano Kit.
3. Compare the Bioanalyzer traces to those traces in Figure 2 and Figure 3. Degraded samples appear as significantly lower intensity traces with the main peak area shifted to the left and typically exhibit much more noise in the trace.

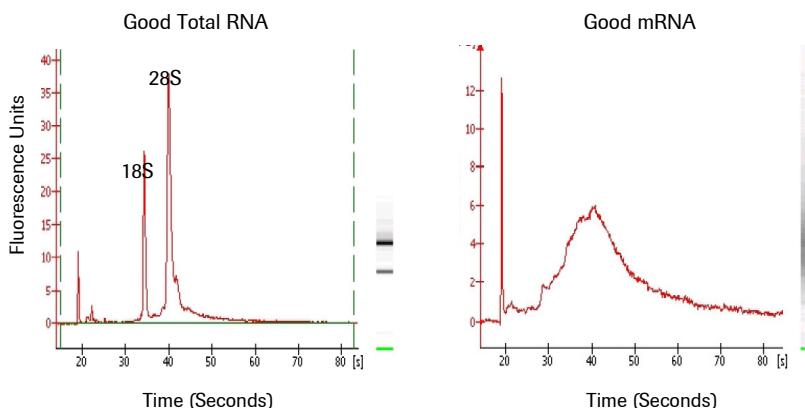


If using a denaturing agarose gel, one reagent source is Ambion NorthernMax reagents for northern blotting. This kit's first section includes reagents and instructions for using denaturing agarose gels. You can also purchase reagents individually. Compare gel images to the Bioanalyzer electropherogram images.

Samples exhibiting degradation should not be used for cDNA synthesis because there is an unacceptable risk of poor results.



**Figure 2: Example Traces for Degraded RNA Samples from a Eukaryotic Organism**



**Figure 3: Example Traces for Non-degraded RNA Samples from a Eukaryotic Organism**



Your traces could look different than these examples. Compare your traces to reference traces from RNA for the organism being researched.

### Step 3. First Strand cDNA Synthesis

Use the Invitrogen SuperScript Double-Stranded cDNA Synthesis Kit to synthesize double-stranded cDNA.

- Thaw and maintain the following components on ice. Combine components in a 0.2 ml tube on ice according to the following tables.



Prepare a 100 pmol/ $\mu$ l primer solution.

Eukaryotic Source of RNA	Total RNA Amount	Poly-A+ RNA (mRNA) Amount
RNA	10 $\mu$ g	1 $\mu$ g
oligo dT Primer	1 $\mu$ l	1 $\mu$ l
DEPC Water*	To volume	To volume
<b>Total</b>	<b>11 <math>\mu</math>l</b>	<b>11 <math>\mu</math>l</b>
Prokaryotic Source of RNA	Total RNA Amount	
RNA	10 $\mu$ g	
Random Hexamer Primer	1 $\mu$ l	
DEPC Water*	To volume	
<b>Total</b>	<b>11 <math>\mu</math>l</b>	

\* Supplied in the SuperScript Double-Stranded cDNA Synthesis Kit.

- Heat sample(s) to 70°C for 10 minutes in a thermocycler. Briefly spin tubes in a microcentrifuge and place them in an ice-water slurry for 5 minutes.
- Add the following to each sample tube. (You can use a master mix when preparing multiple samples.)

Component	Volume
Reaction volume from step 2	11 $\mu$ l
5X First Strand Buffer	4 $\mu$ l
0.1M DTT	2 $\mu$ l
10mM dNTP Mix	1 $\mu$ l
<b>Total</b>	<b>18 <math>\mu</math>l</b>

- Mix gently (avoid vortexing). Briefly spin the tube(s) in a microcentrifuge.
- Place sample(s) in a thermocycler set at 42°C for 2 minutes.
- Add 2  $\mu$ l of SuperScript II RT and mix gently (avoid vortexing).
- Incubate the sample(s) at 42°C for 60 minutes.
- STOP POINT:** Briefly spin the tube(s) in a microcentrifuge. Place the sample(s) on ice until the second strand synthesis. You can store the sample(s) overnight at -20°C.

## Step 4. Second Strand cDNA Synthesis

- Add the following components to the first strand reaction(s) in the indicated order; you can use a master mix. Keep tube(s) on ice or in a PCR tube chiller rack.

Component	Volume
Reaction volume from Step 3.8	20 µl
DEPC Water	91 µl
5X Second Strand Buffer	30 µl
10 mM dNTP Mix	3 µl
10 U/µl DNA Ligase	1 µl
10 U/µl DNA Polymerase I	4 µl
2 U/µl RNase H	1 µl
<b>Total</b>	<b>150 µl</b>

- Mix gently (avoid vortexing). Briefly spin the tube(s) in a microcentrifuge. Incubate at 16°C for 2 hours.
- Add 2 µl of 5 U/µl T4 DNA polymerase to each reaction. Incubate at 16°C for an additional 5 minutes. Do not allow the reaction temperature to exceed 16°C during this step.
- STOP POINT:** Place the sample(s) on ice or in a PCR tube chiller rack, and add 10 µl of 0.5 M EDTA. You can store samples overnight at -20°C.

## Step 5. RNase A Cleanup

- Add 1 µl of 4 mg/ml RNase A solution to the tubes from Step 4.4.



Use caution when working with RNase A. Change gloves after use. Use RNaseZap to clean work area surfaces.

- Mix gently (avoid vortexing). Briefly spin the tube(s) in a microcentrifuge.
- Incubate sample(s) at 37°C for 10 minutes.
- During incubation, centrifuge Phase Lock tube(s) at 12,000 x g for 2 minutes. Label one Phase Lock tube and two 1.5 ml centrifuge tubes for each sample with sample names.
- Add 163 µl of phenol:chloroform:isoamyl alcohol to one set of 1.5 ml centrifuge tubes.
- Transfer the sample(s) to the tube(s) containing phenol:chloroform:isoamyl alcohol. Vortex well.
- Transfer samples with the phenol:chloroform:isoamyl alcohol to Phase Lock tubes.
- Centrifuge at 12,000 x g for 5 minutes.
- Transfer the upper, aqueous layer to a clean, labeled 1.5 ml tube.

## Step 6. cDNA Precipitation

1. Add 16  $\mu$ l (0.1 volume of Step 5.9) of 7.5 M ammonium acetate to the samples. Mix by repeated inversion. Briefly spin the tube(s) in a microcentrifuge.
2. Add 7  $\mu$ l of 5 mg/ml glycogen to the samples. Mix by repeated inversion. Briefly spin the tube(s) in a microcentrifuge.
3. Add 326  $\mu$ l (2 volumes of Step 5.9) of ice-cold absolute ethanol to the samples. Mix by repeated inversion.
4. Centrifuge at 12,000 x g for 20 minutes.
5. Remove supernatant. Take care not to disturb the pellet.
6. Add 500  $\mu$ l of ice-cold 80% ethanol (v/v). Mix by repeated inversion.
7. Centrifuge tubes at 12,000 x g for 5 minutes.
8. Remove supernatant. Take care not to disturb the pellet.
9. Repeat steps 6 - 8.
10. Dry the pellet in a DNA vacuum concentrator.
11. Rehydrate samples with 20  $\mu$ l of VWR water.

## Step 7. Spectrophotometric QC of cDNA

1. Quantitate each cDNA sample according to the following formula:

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

2. Verify that all samples meet the following requirements:

- Concentration  $\geq$  100 ng/ $\mu$ l
- $A_{260}/A_{280} \geq 1.8$
- $A_{260}/A_{230} \geq 1.8$

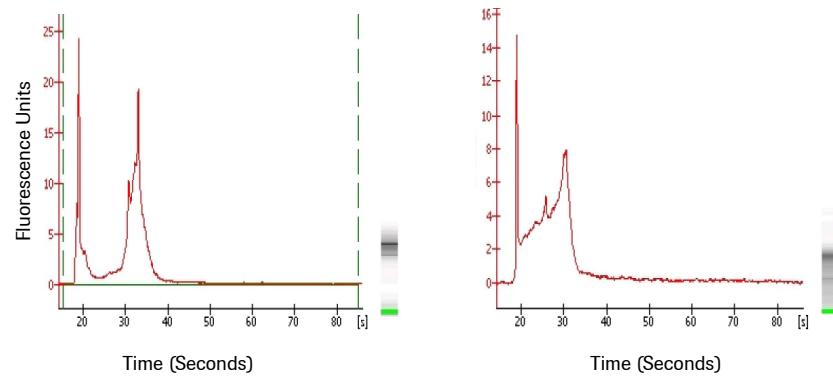
## Step 8. Bioanalyzer/Gel QC of cDNA

1. Transfer 250 ng cDNA to a sterile microcentrifuge tube. Store the remainder of your sample on ice or at -20°C.
2. Analyze the samples using the Agilent Bioanalyzer and RNA 6000 Nano Kit.
3. Compare the Bioanalyzer traces to the traces displayed below. Verify that all samples meet the following requirement for acceptance:
  - Median size  $\geq$  400 bp when compared to a DNA ladder.
  - Looks similar to the examples of good cDNA sample traces displayed below.



If using an agarose gel, compare the gel images to the Bioanalyzer's electropherogram images.

Samples exhibiting degradation should not be carried through labeling and hybridization because there is an unacceptable risk of poor results.



**Figure 4: Example Traces Showing Good cDNA from a Eukaryotic Organism**



Your traces could look different than these examples. Compare your traces to reference traces for the organism being researched.

# Chapter 3. Sample Labeling

Chapter 3 describes how to label your cDNA samples using a NimbleGen One-Color DNA Labeling Kit.

Be aware of the following when using these kit contents:

- Aliquot dNTPs and Cy primer into single-use amounts.
  - 5 M NaCl could precipitate. Vortex or heat if necessary.
1. Prepare the following solution in a 1.5 ml microfuge tube:

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

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

2. Briefly centrifuge Cy3-Random Nonamers (vial 3) because some of the product could have dislodged during shipping. Dilute the primer in 924 µl of Random Primer Buffer with β-Mercaptoethanol. Aliquot 40 µl individual reaction volumes in 0.2 ml thin-walled PCR tubes and store at -20°C, protected from light.



Do not store diluted primers longer than 4 months.

3. Assemble the following components in separate 0.2 ml thin-walled PCR tubes:

Component	All Array Formats
cDNA	1 µg
Diluted Cy3-Random Nonamers from step 2	40 µl
Nuclease-free Water (vial 1)	To volume
<b>Total</b>	<b>80 µl</b>

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



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

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



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

dNTP/Klenow Master Mix: Recipe per Sample	All Array Formats
10mM dNTP Mix (vial 5)	10 $\mu$ l
Nuclease-free Water (vial 1)	8 $\mu$ l
Klenow Fragment (3'->5' exo-) 50U/ $\mu$ l (vial 4)	2 $\mu$ l
<b>Total</b>	<b>20 <math>\mu</math>l</b>

- 6.** Add 20  $\mu$ l 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
Reaction volume from step 4	80 $\mu$ l
dNTP/Klenow Master Mix	20 $\mu$ l
<b>Total</b>	<b>100 <math>\mu</math>l</b>

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



Do not vortex after addition of Klenow.

- 8.** Quick-spin to collect contents in bottom of the tube.  
**9.** Incubate for 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
Reaction volume from step 6	100 $\mu$ l
Stop Solution (0.5M EDTA) (vial 6)	10 $\mu$ l
<b>Total</b>	<b>110 <math>\mu</math>l</b>

- 11.** Add 5M NaCl to each tube.

Component	All Array Formats
Reaction volume from step 10	110 $\mu$ l
5M NaCl (vial 7)	11.5 $\mu$ l
<b>Total</b>	<b>121.5 <math>\mu</math>l</b>

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

Component	All Array Formats
Reaction volume from step 11	121.5 $\mu$ l
Isopropanol	110 $\mu$ l
<b>Total</b>	<b>231.5 <math>\mu</math>l</b>

- 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.  
**15.** Rinse pellet with 500  $\mu$ l 80% ice-cold ethanol. Dislodge pellet from tube wall by pipetting a few times.

- 16.** Centrifuge at 12,000 x g for 2 minutes. Remove supernatant with a pipette.
- 17.** Dry contents in a DNA vacuum concentrator on low heat until dry (approximately 5 minutes), protected from light.
- 18. STOP POINT:** Proceed to step 19, or store labeled samples at -20°C (up to 1 month), protected from light.
- 19.** Spin tubes briefly prior to opening. Rehydrate each pellet in 25 µl Nuclease-free Water (vial 1).
- 20.** Vortex for 30 seconds and quick-spin to collect contents in bottom of the tube. Continue to vortex or let sit at room temperature, protected from light, for approximately 5 minutes or until the pellet is completely rehydrated, then vortex again and quick-spin.
- 21.** Quantitate each sample using the following formula:

Concentration (µg/ml) = A<sub>260</sub> x 50 x Dilution Factor



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

- 22.** Based on the concentration, calculate the volume of Cy3-labeled cDNA sample required for each hybridization per the following table and aliquot in a 1.5 ml tube:

Sample Requirements*	385K Array	4x72K Array	12x135K Array
Cy3-labeled cDNA Sample	Prok: 3 µg Euk: 6 µg	Prok: 2 µg Euk: 4 µg	Prok: 2 µg Euk: 4 µg

\* Prok = Prokaryote; Euk = Eukaryote

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

## 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 dye, which are sensitive to photobleaching and freeze-thawing. After thawing stock tubes for the first time, aliquot the Alignment Oligo and STCs into single-use volumes and freeze at -20°C. Protect tubes from light.

## Step 1. Prepare Samples

1. Set the Hybridization System to 42°C. With the cover closed, allow at least 3 hours for the temperature to stabilize.



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

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



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

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

3. Vortex well and spin to collect contents in bottom of the tube.
4. Using components from a NimbleGen Hybridization Kit, prepare the hybridization solution master mix according to the following table. For 4x72K and 12x135K arrays, the amount listed is sufficient to hybridize all arrays on one slide. To hybridize multiple slides, adjust the amounts accordingly.

Hybridization Solution Master Mix to Hybridize a Single Slide	385K Array	4x72K Array	12x135K Array
2X Hybridization Buffer (vial 1)	11.8 µl	29.5 µl	88.5 µl
Hybridization Component A (vial 2)	4.7 µl	11.8 µl	35.4 µl
Alignment Oligo (vial 3)	0.5 µl	1.2 µl	3.6 µl
<b>Total</b>	<b>17 µl</b>	<b>42.5 µl</b>	<b>127.5 µl</b>

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

Component	385K Array	Each Sample for a 4x72K Array	Each Sample for a 12x135K Array
Resuspended sample from step 2	5 µl	3.3 µl	3.3 µl
Hybridization solution from step 4	13 µl	8.7 µl	8.7 µl
<b>Total</b>	<b>18 µl</b>	<b>12 µl</b>	<b>12 µl</b>

6. Vortex well (approximately 15 seconds) and spin to collect contents in bottom of the tube. Incubate at 95°C for 5 minutes, protected from light.
7. Place tubes at 42°C (in the Hybridization System sample block or heat block) for at least 5 minutes or until ready for sample loading. Vortex and spin prior to loading.

## Step 2. Prepare Mixers

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



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



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

Array Format	Mixer
385K array	X1 mixer
4x72K array	X4 mixer
12x135K array	HX12 mixer

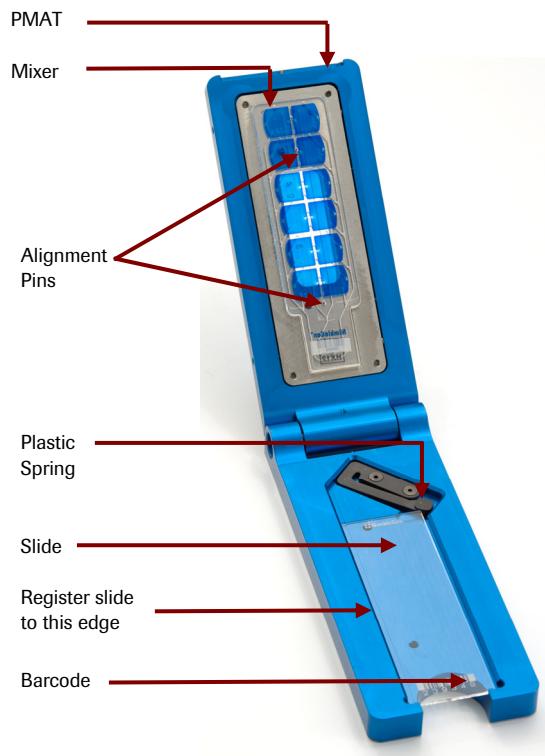
2. Position the Precision Mixer Alignment Tool (PMAT) with its hinge on the left. Open the PMAT (Figure 5).
3. Snap the mixer onto the two alignment pins on the lid of the PMAT with the tab end of the mixer toward the inside hinge and the mixer's adhesive gasket facing outward (Figure 5).

4. While pushing back the plastic spring with a thumb, place the slide in the base of the PMAT so that the barcode is on the right and the corner of the slide sits against the plastic spring. The NimbleGen logo and barcode number should be readable. Remove your thumb and make sure the spring is engaging the corner of the slide and the entire slide is registered to the edge of the PMAT to the rightmost and closest to you. In addition, be sure that the slide is lying flat against the PMAT. Gently blow compressed nitrogen or argon gas across the mixer and slide to remove dust.



Take care to align the slide correctly in the PMAT. Incorrectly aligned slides may result in inaccurate attachment of the mixer and may affect the array features, or may not fit well into the Mixer Disassembly Tool used to remove the mixers after hybridization.

5. Using forceps, remove the backing from the adhesive gasket of the mixer and close the lid of the PMAT so that the gasket makes contact with the slide.
6. Lift the lid by grasping the long edges of the PMAT while simultaneously applying pressure with a finger through the window in the lid of the PMAT to free the mixer-slide assembly from the alignment pins.



**Figure 5: PMAT with HX12 Mixer and Slide.** For photographic purposes only, blue coloring was used to show the location of the mixer's hybridization chambers. The hybridization chambers of the mixer you receive will not be blue.

7. Remove the mixer-slide assembly from the PMAT.
8. Place the mixer-slide assembly on the back of a 42°C heating block for 5 minutes to facilitate adhesion of the mixer to the slide.

9. Rub the Mixer Brayer over the mixer with moderate pressure to adhere the adhesive gasket and remove any bubbles. For X1 mixers, start in the center of the array and rub outwards. For X4 and HX12 mixers, first use a corner of the Mixer Brayer to rub the borders between the arrays and then rub around the outside of the arrays. The adhesive gasket will become clear when fully adhered to both surfaces.
10. Place the mixer-slide assembly in the slide bay of the Hybridization System.

### Step 3. Load & Hybridize Samples

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

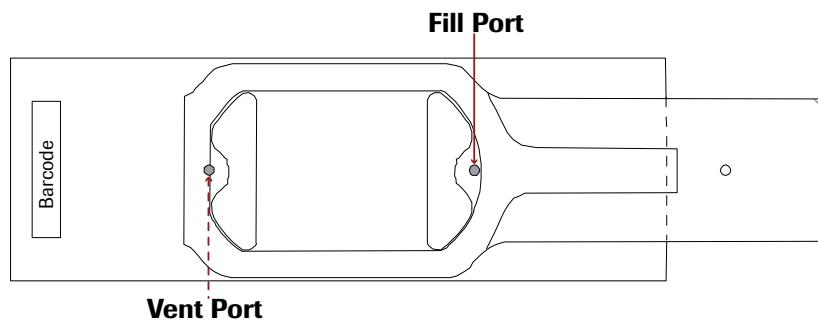


Figure 6: X1 Mixer and Slide for a 385K Array

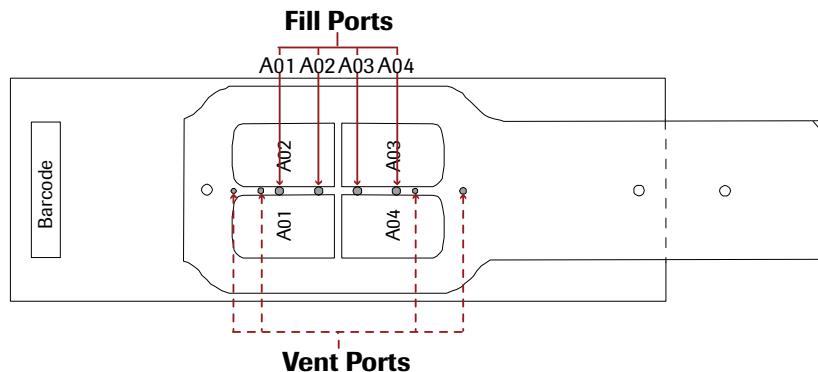
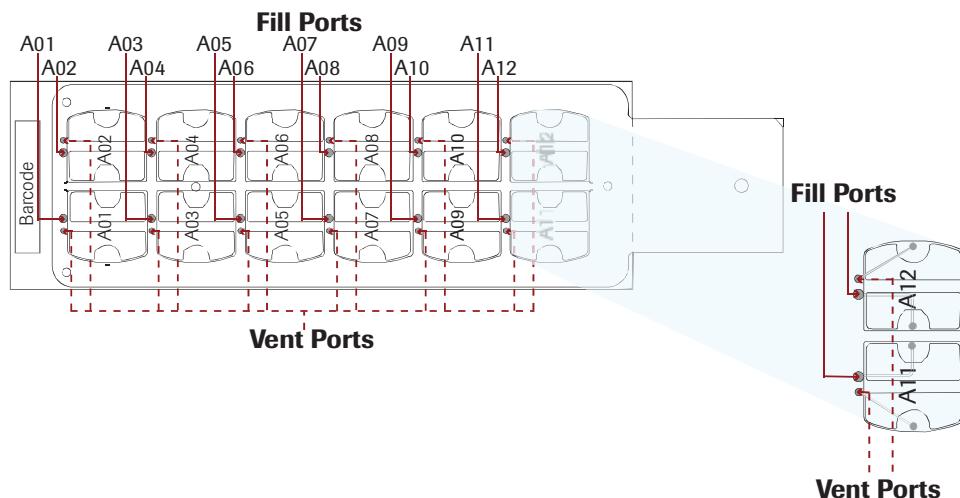


Figure 7: X4 Mixer and Slide for a 4x72K Array



**Figure 8: HX12 Mixer and Slide for a 12x135K Array**

2. Keep the following in mind before loading sample:
  - Leave residual volume in the sample tube to avoid bubbles. The volumes listed in the table below account for this additional amount.
  - After aspirating the designated sample volume, inspect the pipette tip for air bubbles. Dispense and reload the pipette if bubbles exist.

Keep the following in mind when loading sample:

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

Component	385K Array	4x72K Array	12x135K Array
Sample Loading Volume	16 $\mu$ l	8 $\mu$ l	6 $\mu$ l
Pipette Tip	CP100	CP10*	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 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.
    - b. Use one mixer port seal to cover the fill port and another to cover the vent port on X1 mixer. 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 a 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. Dry any overflow from the fill and vent ports with a cotton swab after loading the array. 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 both the fill and vent ports on the mixers, filling and sealing one chamber at a time. Press the mixer port seal, using uniform pressure across the seal to adhere.
-  Mixer port seals are now used to seal fill and vent ports on HX12 mixers instead of mixer multi-port seals.
- c. Use forceps to press the mixer port seal around the fill and vent ports to ensure it is adhered in those areas.
4. Close the bay clamp.
  5. Turn on the Mixing Panel on the Hybridization System, set the mix mode to B, and press the mix button to start mixing. Confirm that the Hybridization System recognizes the slide in each occupied bay (its indicator light becomes green).
  6. Approximately 10 minutes after starting the Hybridization System:
    - Ensure the mix mode is set to B.
    - Ensure a green light is displayed for all occupied stations.
  7. Hybridize sample at 42°C to the array(s) for 16 - 20 hours.

## Step 4. Wash Hybridized Arrays



To ensure high quality data, it is important to proceed through all the washing and drying steps without interruption. The NimbleGen Microarray Dryer dries up to 24 slides at a time. If using a microarray dryer that dries one slide at a time, wash only one slide at a time.

1. Locate the components of the NimbleGen Wash Buffer Kit and NimbleGen Array Processing Accessories (refer to page 9).
- 
- Prior to the first use of the Wash Buffer Kit, reconstitute the DTT. In a fume hood, prepare 1 M DTT solutions by adding 1.2 ml of water (vial 5) to each tube of dry DTT (vial 4). After reconstitution, store the 1 M DTT solutions at -15°C to -25°C.
2. Before removing the mixer-slide assemblies from the Hybridization System, prepare Washes I, II, and III according to the following tables. Note that you prepare two containers of Wash I.

Washing Multiple Slides	Wash I (user-supplied dish <sup>1</sup> )	Washes I, II, and III (wash tank <sup>2</sup> )
	Total	270 ml
Washing One Slide	Wash I (user-supplied dish <sup>1</sup> )	Washes I, II, and III (slide container <sup>2</sup> )
	Total	270 ml

1. Ensure that this dish is shallow and wide enough to accommodate the mixer-slide assembly loaded in the Mixer Disassembly Tool. This dish must also be small enough to ensure that the Mixer Disassembly Tool is completely submerged in the wash solution.
2. If washing multiple slides, prepare the washes in the wash tanks. If washing only one slide, prepare the washes in the slide containers.
3. To facilitate the removal of the mixer, heat the shallow dish containing Wash I to 42°C. Roche NimbleGen recommends measuring the temperature of Wash I at every use. Keep the remaining three wash solutions at room temperature.
4. Insert the Mixer Disassembly Tool into the shallow dish containing warm Wash I. If you will be washing multiple slides, insert a slide rack into the wash tank containing Wash I at room temperature.
5. Remove a mixer-slide assembly from the Hybridization System and load it into the Mixer Disassembly Tool immersed in the shallow dish containing warm Wash I.

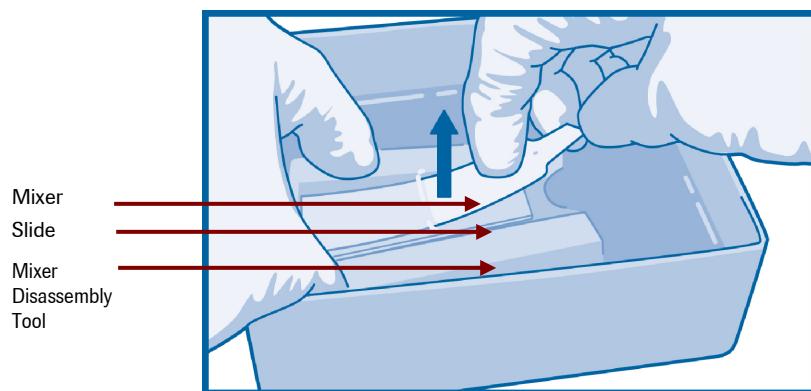


Do not allow the mixer-slide assembly to cool before removing the mixer. Keep power on to the Hybridization System's heat block and mixer system during mixer-slide disassembly, and transfer each mixer-slide assembly one at a time to Wash I for immediate removal of the mixer.

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



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



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

- Working quickly, discard the mixer and remove the slide from the Mixer Disassembly Tool.
- Gently agitate the slide for 10 - 15 seconds in the shallow dish containing warm Wash I to quickly remove the hybridization buffer.

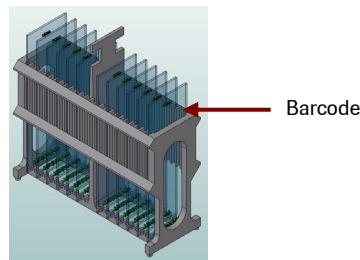


It is important for achieving good array uniformity to quickly and evenly wash the hybridization buffer off the slide surface as soon as the mixer is removed.

- 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 wet at all times during all wash steps.



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



If you are using a NimbleGen Microarray Dryer or other microarray dryer that dries multiple slides at a time, repeat steps 4 - 9 until you have removed the mixer from all slides to wash. Load each slide into the slide rack with the array facing the same direction.

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



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

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



Do not allow slides to dry between wash steps.

- 12.** Transfer the slide(s) to Wash III and wash for 15 seconds with vigorous, constant agitation. If washing multiple slides using the slide rack, rock the wash tank so the wash solution covers and cleans the tops of the slide(s).

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

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



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

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

# Notes

# Chapter 5. One-Color Array Scanning

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

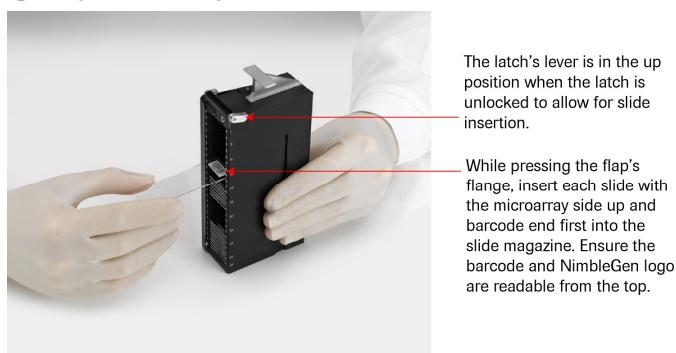
Before starting these procedures, review the information in the *NimbleGen MS 200 Microarray Scanner Operator's Manual* (available at [www.nimblegen.com/products/instruments/](http://www.nimblegen.com/products/instruments/) under Literature) or the online help available via the MS 200 Data Collection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



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

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

1. Start the control unit and log into your user account as msOperator or other account as set up by your system administrator.  
Account: msOperator  
Password: 1-msOperator
2. Turn on the scanner using the power switch on the left side.
3. Using a compressed gas nozzle, gently blow compressed nitrogen or argon gas across the slide to remove any dust or debris from the array. Do not use canned aerosol compressed air for this purpose.
4. Insert slides into the Slide Magazine as described in Figure 11. Numbered slots provide spacing for inserting slides.



**Figure 11: Inserting Slides into the Slide Magazine**

5. Press the insert/eject magazine button on the scanner to open the stacker cover. Insert the slide magazine with loaded slides, aligning the slot on the slide magazine's side to join with the rail profile inside the scanner. The lowering of the slide magazine is interrupted by a mechanical hold point. Apply gentle pressure to complete insertion. Press the insert/eject magazine button to close the stacker cover (Figure 12). The initialization process starts, checking the slide magazine to determine which slots are occupied.



Figure 12: Inserting the Slide Magazine into the Scanner

## Step 2. Start the Software & Turn on the Lasers

1. Double-click the NimbleGen MS200 icon to launch the MS 200 Data Collection Software. Make sure that the software has completely loaded before continuing.
2. Click the Green Laser button in the Laser Control (Figure 13) to switch on the Channel 1 laser if scanning only one-color NimbleGen Gene Expression arrays. Or, click both the Green Laser and Red Laser buttons if scanning both one- and two-color arrays. Allow laser(s) to warm for 10 minutes.

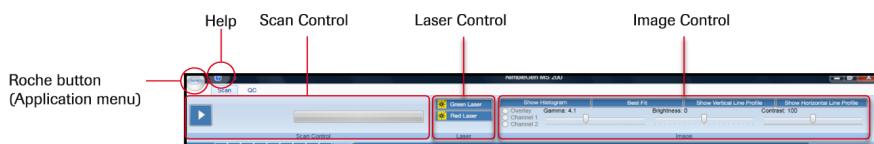


Figure 13: Top of Data Collection Workspace, showing Scan Control, Laser Control, and Image Control

3. Review the Magazine Control (Figure 14) in the Data Collection Software. Ensure that a green box appears in the *Slide Present* field for each slide loaded into the slide magazine.

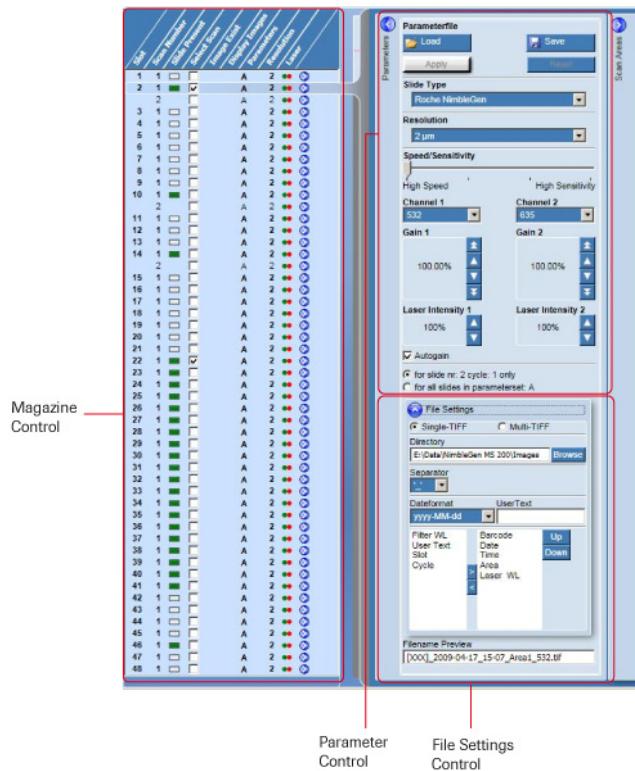


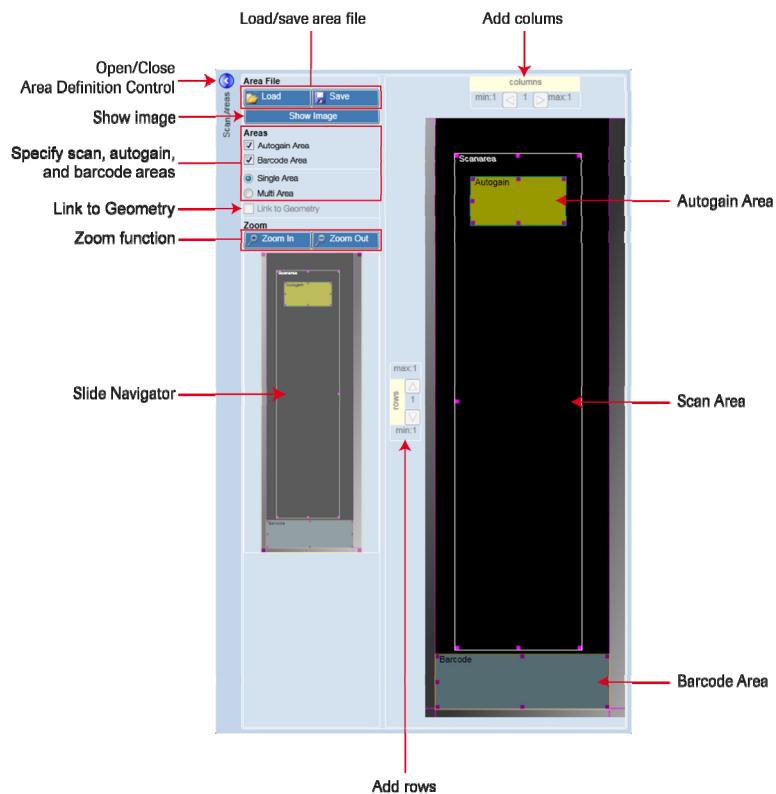
Figure 14: Magazine Control, Parameter Control, and File Setting Control

### Step 3. Set Scan Parameters Using the Software

1. Use the Parameter Control (Figure 14) to set the parameters to use when scanning:
  - a. To open the Parameter Control if not displayed, go to the row of a slide to scan in the Magazine Control and click its Open Parameter Control button (●) to display the Parameter Control.
  - b. Do not adjust the following default parameters:
    - Slide type
    - Channel 1
    - Laser Intensity 1 and Laser Intensity 2
    - for slide nr: 2 cycle: 1 only
  - c. For Channel 2, select “none.”
  - d. Adjust the following default parameters if necessary:
    - Resolution
    - Speed/Sensitivity
    - Autogain
  - e. To save any changes to the settings, click **Apply**.
  - f. (Optional) Click **Save** to save settings to a parameters file, which allows the settings to be applied to other slides.
  - g. (Optional) To process the slide multiple times using the same or different parameters, create up to 12 cycles.

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

2. Use the File Settings Control (Figure 14) to specify image file settings:
  - a. To open the File Settings Control if not displayed, go to the row of a slide to scan in the Magazine Control and click its **Open Parameter Control** button (❸) (Figure 14). In the Parameter Control, click the **Open/Close File Settings** button (❹) to open the File Settings Control.
  - b. Choose the **Single-TIFF** option button to generate one image file in Tagged Image File Format (TIFF, .tif) per channel.
  - c. If necessary, change the path to which the images files will be saved. The default path is E:\Data\NimbleGen MS 200. To change the path, click **Browse** to open a dialog box to specify a location in the directory and click **OK** to confirm.
  - d. Use the annotation list to add or change annotations to include in file names. Click an annotation in the left list box and then click the right arrow button (❺) to add to the right list box.  
If you will be using NimbleScan software for data analysis, specify and order the annotations as follows:  
`<Barcode>_<User Text>_<Laser WL>.tif`  
where “WL” means wavelength. To change the order of the annotations, select the annotation and click the **Up** or **Down** button. For “User Text,” make sure to type the text in the *User Text* field.  
The *Filename Preview* text box of the Parameter Control displays the entire naming convention of the image file (.tif).
3. Use the Area Definition Control (Figure 15; denoted as *Scan Areas* in the software interface) to set scan, barcode, and autogain areas.
  - a. To open the Area Definition Control if not displayed, in the Parameter Control, click the **Open/Close Area Definition Control** button (❻) (shown on the right side of Figure 14 above the *Scan Areas* label in the software interface). Figure 15 shows the components of the Area Definition Control.

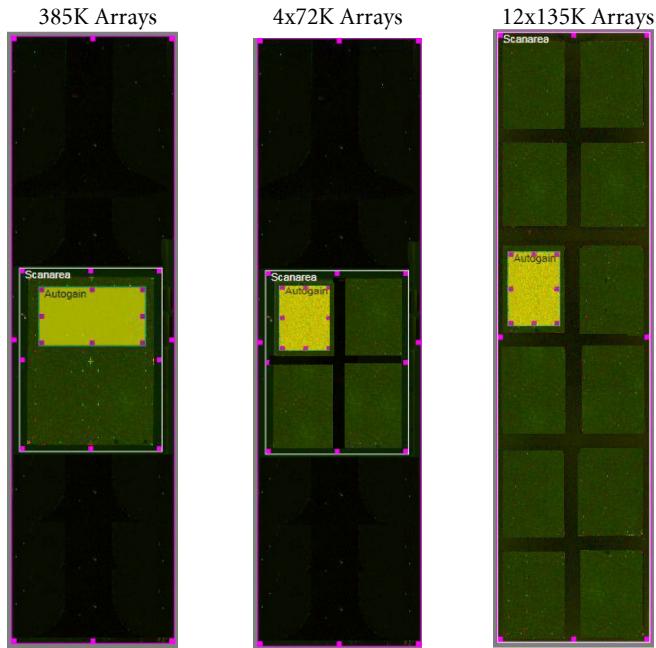
**Figure 15: Components of the Area Definition Control**

- b.** Ensure the following are selected:
- *Barcode area* checkbox
  - *Autogain area* checkbox, if the Autogain checkbox was selected in the Parameter Control (Figure 14)
  - *Single Area* option - this is the preferred option when scanning single and multiplex NimbleGen arrays. For multiplex arrays, you will use NimbleScan software's burst functionality to create individual image files for multiplex arrays.

- c.** Review and if necessary adjust the scan area:
- For NimbleGen 12x135K arrays, use the default selection for the scan area.
  - (Optional) For NimbleGen 385K and 4x72K arrays, reduce the scan area, which will reduce the scan time. To define the scan area, download and use area files available at [www.nimblegen.com/scanner](http://www.nimblegen.com/scanner) under *Download*. These files identify scan areas that are specific for NimbleGen 385K or 4x72K arrays. For instructions on how to use area files or how to manually adjust the scan area, refer to the *NimbleGen MS 200 Microarray Scanner Operator's Manual*.
  - If desired, click *Save* to save settings to an area file for future use.

- d.** Review and if necessary adjust the autogain area.

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



**Figure 16: Examples of Autogain Areas for NimbleGen Arrays**

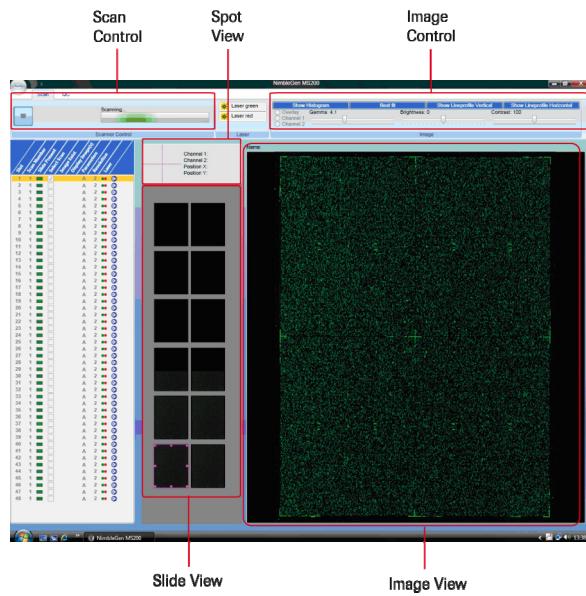
4. Repeat the instructions above for each slide to process during the experiment.

If you saved parameter file(s) and area file(s), click **Load** in the Parameter Control or Area Definition Control, respectively, to select a file and then click **Apply** to confirm your selection.

#### Step 4. Scan the Slides

1. Click the Start/Stop Scan button (▶) in the Scan Control (Figure 17).
2. When prompted, specify the folder and file naming to save the session file.

The scan process is then initiated. The Parameter Control and Area Definition Control close. The Image View, Slide View, and Spot View open (Figure 17), and the Scan Control shows the current task that is being performed above the progress bar.

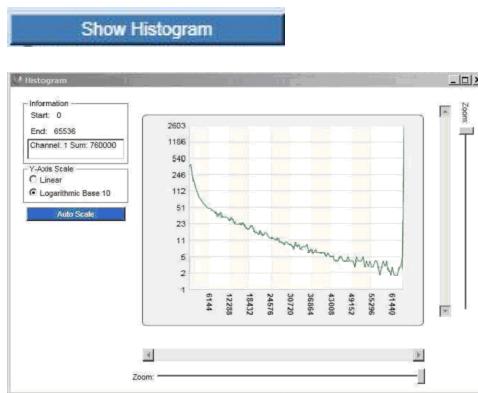
**Figure 17: Example of a Run Time Window**

3. (Optional) To manually adjust the PMT gain while scanning at 5  $\mu\text{m}$  or higher resolution:

- a. Use the *Gain 1* spin box that appears in the Scan Control (Figure 17) to adjust the gain.



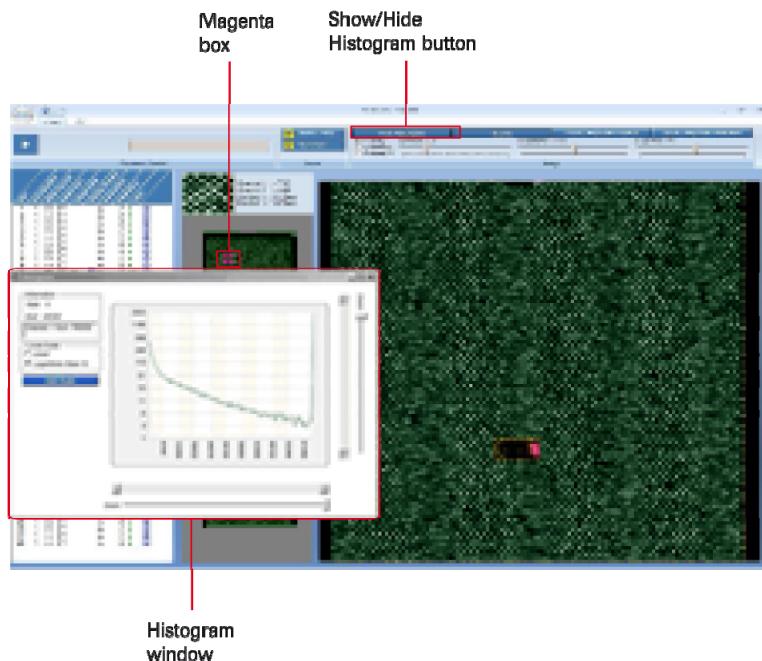
- b. Click *Show Histogram* in the Image Control (Figure 17) to view the histogram. Use the zoom feature (magenta box in the Slide View, Figure 19), to select where to view the histogram.

**Figure 18: Histogram Window**

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

## Step 5. Review Scanned Images

- Once the scan of a slide is completed, a blue Displays Images button ( appears in the *Display Images* field of the Magazine Control (Figure 14). Move the mouse pointer over the button to display the directory location and file name of the acquired image. Click the blue Displays Images button () to display the image (both images) in the Image View.
- To view a small region of the array and to select where to view the histogram (Figure 19), reduce the size of the magenta box that outlines the slide image in the Slide View so that the magenta box surrounds the area of interest. The area of interest will appear in the Image View. Click Show/Hide Histogram to display the histogram.



**Figure 19: Example Window Showing Magenta Box in the Slide View, Show/Hide Histogram Button, and Histogram Window**

- Adjust contrast and brightness using the Image Control (Figure 20) to provide an improved view of features on the image. Adjusting these settings allows faint features to be more easily seen.



**Figure 20: Image Control**

# Chapter 6. NimbleScan Data Analysis

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

## Step 1. Burst Multiplex Image (4x72K & 12x135K Arrays Only)

If your files contain the scanned images of 4x72K or 12x135 arrays, follow the steps below to burst (separate) each slide image into separate array images. Otherwise, proceed to "Step 2. Import Image."

1. Select File -> Burst Multiplex Image. The Burst Multiplex Image dialog box appears.
2. To choose the .tif files to burst, click Add images.
3. Navigate to the directory containing your array image file (.tif) and select the file.
4. Click Add to batch. Multiple slide images can be burst at once.
5. Browse to select the correct multiplex description file (.ncd) on the Design Information disk provided with the 4x72K or 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 image files from a 4x72K array image or 12 image files from a 12x135K array image. The array designation (A01, A02, etc.) for each bursted image is specified in the original image's file name. For example:
  - <NNNNNA01>\_<XXXXX>\_532.tif...<NNNNNA04>\_<XXXXX>\_532.tif
  - <NNNNNA01>\_<XXXXX>\_532.tif...<NNNNNA12>\_<XXXXX>\_532.tifwhere NNNNN is the slide's barcode number and XXXXX is optional user-defined text.
8. Repeat steps 1 - 7 for each multiplex image.



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

## Step 2. Import Image

1. Select File -> Open. The Open an Alignment dialog box appears.
2. Navigate to the directory containing your array images and select one image.
3. Select the design file (.ndf) in the Design Information disk provided with your array. This file describes the placement of the probes on the array.
4. Select the gene description file (.ngd) in the Design Information disk provided with your array. This file contains NimbleGen sequence identifiers as well as information about the sequence.
5. Click Open.

### 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.



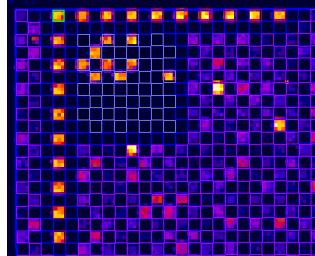
If the software indicates auto alignment was not successful, perform a manual alignment as described in the *NimbleScan Software User's Guide*.

3. Zoom into the top corner of the array with the zoom tool.

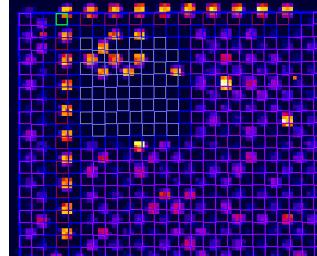


Check to make sure all fiducial controls align correctly with the grid.

Good Alignment



Poor Alignment

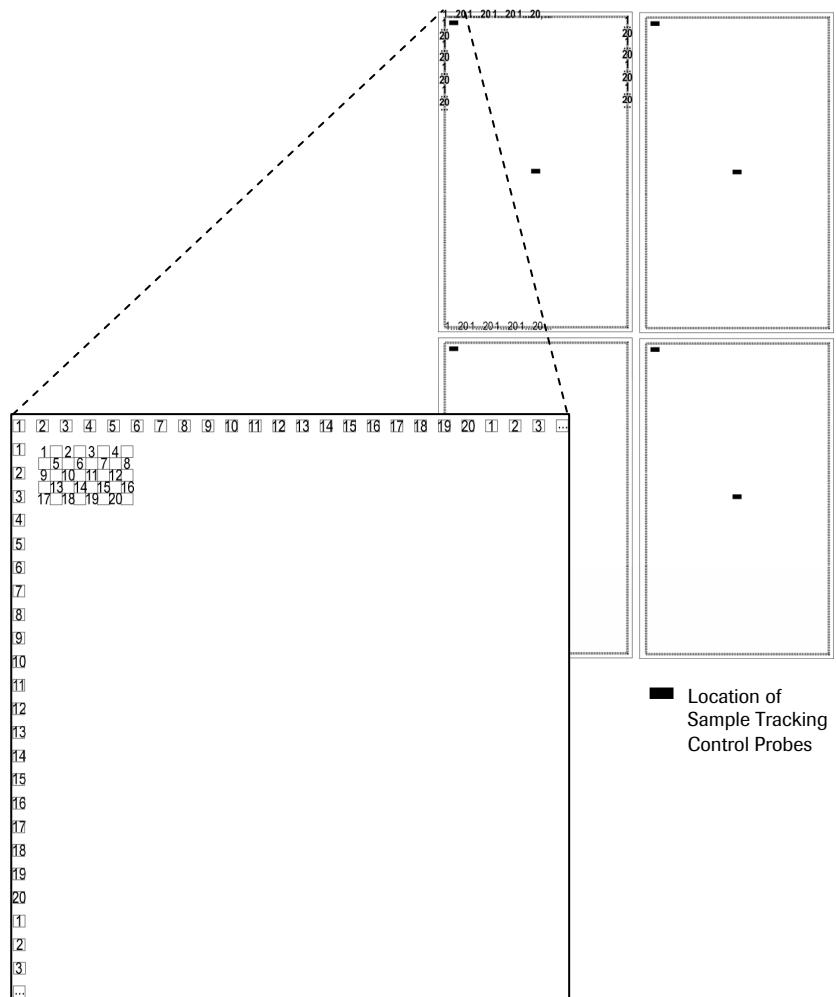


**Figure 21: Examples Showing Good and Poor Grid Alignments**

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.
5. Move to each corner using the jump to location buttons. For 385K arrays, also check the center fiducial controls.
6. Select the local alignment tool to fine-tune the alignment.
7. Select File -> Save to save the gridded image.
8. If you are analyzing data from a 4x72K or 12x135K array, proceed to "Step 4. Confirm Experimental Integrity (4x72K and 12x135K Arrays Only)." If you are analyzing data from a 385K array, proceed directly to "Step 5. Generate an Experimental Metrics Report."

### Step 4. Confirm Experimental Integrity (4x72K & 12x135K Arrays Only)

A unique STC should have been added to each sample prior to loading onto 4x72K 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 22). Roche NimbleGen recommends performing a sample tracking analysis (refer to page 44) and visually checking the STC features along the perimeter (refer to page 46) to confirm that the correct sample has been added to each array.



**Figure 22: 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 with Sample Tracking Control probes (Figure 22). 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 23.
  - Confirm that the Image ID, Design ID, and Design Name are accurate.
  - Enter the Sample Name and Input STC in the appropriate spreadsheet cells. The Input STC is the unique STC number added to each sample before hybridization.
  - For each array, review the signal intensity and the Absent or Present call for each of the up to 20 STCs that Roche NimbleGen may provide. The STC identified as Present should be the same as the Input STC you entered for the sample.
  - Ensure that a Present call is reported for only the unique STC added to the sample. If more than one Present call is reported, the integrity of your data could be compromised due to cross-contamination that occurred during sample preparation, loading, or hybridization. The extent of cross-contamination that can be tolerated depends on your samples, experimental setup, and experimental goals. Roche NimbleGen recommends repeating experiments that show cross-contamination.
  - Select File -> Save to save the changes to the file.

Image ID	109037A01_510_532	109037A02_510_532	109037A03_510_532	109037A04_510_532
Design ID	5010	5010	5010	5010
Design Name	HG18_60mer_expr	HG18_60mer_expr	HG18_60mer_expr	HG18_60mer_expr
Sample Name				
Array Number	A01	A02	A03	A04
Input STC				
STC 1 Intensity	39528	210	210	218
STC 2 Intensity	215	228	253	207
STC 3 Intensity	182	48920	232	206
STC 4 Intensity	191	214	221	250
STC 5 Intensity	195	248	40937	244
STC 6 Intensity	183	242	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

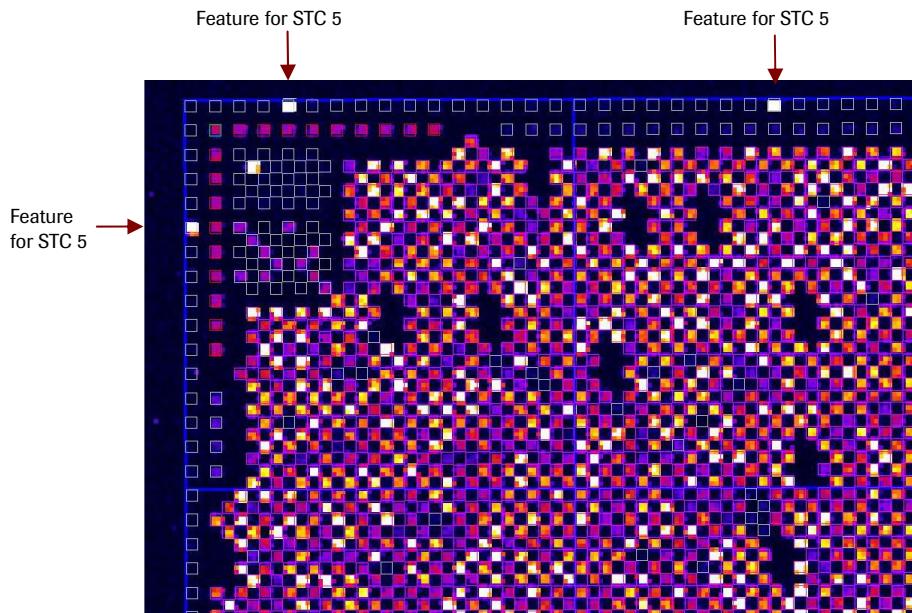
**Figure 23: Example of a Sample Tracking Report**

### 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 24 shows an example of an array hybridized with a sample containing STC 5.



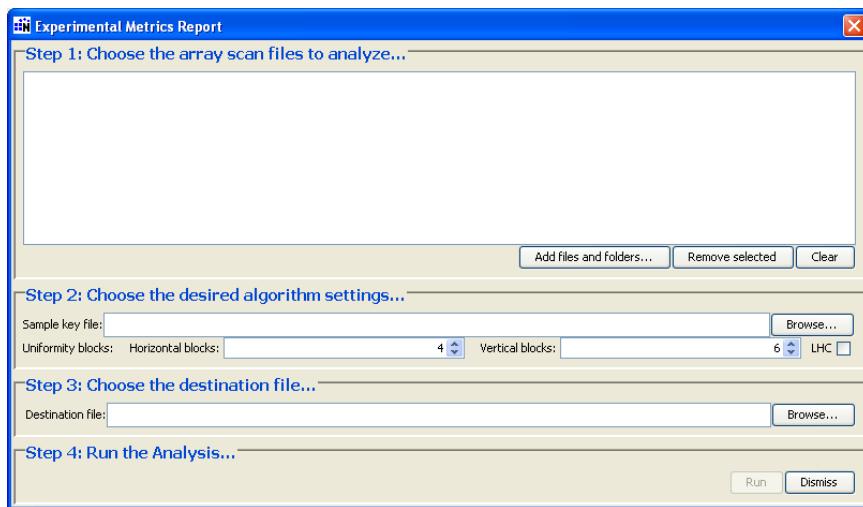
**Figure 24: Example of an Array Hybridized with Sample Containing STC 5 on a 4x72K Array**

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

### Step 5. Generate an Experimental Metrics Report

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

1. Select Analysis -> Generate Experimental Metrics Report.

**Figure 25: 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).
  - Do not select the *LHC* checkbox. This checkbox is intended for two-color NimbleGen microarray experiments that used the NimbleGen Labeling and Hybridization Kit.
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 26.

IMAGE_NAME	INTER_DENSITY	QUARTILE_MEAN	RATIO_RANGE	SIGNAL_MEAN	UNIFORMITY_MEAN	UNIFORMITY_CV	NUM_EMPTY	MEAN_EMPTY	NUM_EXPERIMENTAL	MEAN_EXPERIMENTAL	NUM_RANDOM	MEAN_RANDOM	LHC_2X	LHC_3X	LHC_4X	SLOPE	LHC_DEL
389720A01_532.tif	2.307	0	0.279	2225.249	0.067	146240	532.228	141240	2225.314	0	0 n/a	n/a	n/a	n/a	n/a	n/a	
389720A02_532.tif	2.091	0	0.559	1585.134	0.151	146240	423.689	141240	1585.028	0	0 n/a	r/a	n/a	n/a	n/a	n/a	
389720A03_532.tif	2.645	0	0.238	2464.229	0.058	146240	509.767	141240	2463.868	0	0 n/a	r/a	n/a	n/a	n/a	n/a	
389720A04_532.tif	2.55	0	0.354	2682.582	0.07	146240	530.008	141240	2684.082	0	0 n/a	r/a	n/a	n/a	n/a	n/a	
389720A05_532.tif	2.611	0	0.281	2695.08	0.068	146240	525.986	141240	2696.733	0	0 n/a	r/a	n/a	n/a	n/a	n/a	
389720A06_532.tif	2.179	0	0.185	1686.449	0.045	146240	399.886	141240	1686.383	0	0 n/a	r/a	n/a	n/a	n/a	n/a	
389720A07_532.tif	2.789	0	0.312	2649.007	0.059	146240	535.421	141240	2649.121	0	0 n/a	r/a	n/a	n/a	n/a	n/a	
389720A08_532.tif	2.571	0	0.251	2650.231	0.058	146240	533.159	141240	2651.372	0	0 n/a	r/a	n/a	n/a	n/a	n/a	
389720A09_532.tif	2.637	0	0.296	2682.982	0.071	146240	518.832	141240	2685.641	0	0 n/a	r/a	n/a	n/a	n/a	n/a	
389720A10_532.tif	2.298	0	0.189	1814.673	0.043	146240	412.587	141240	1814.983	0	0 n/a	r/a	n/a	n/a	n/a	n/a	
389720A11_532.tif	2.298	0	0.259	2011.69	0.067	146240	462.613	141240	2011.819	0	0 n/a	r/a	n/a	n/a	n/a	n/a	
389720A12_532.tif	2.266	0	0.362	1974.577	0.062	146240	425.802	141240	1975.983	0	0 n/a	r/a	n/a	n/a	n/a	n/a	

Figure 26: Example of an Experimental Metrics Report

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

- *IMAGE\_NAME*. The name of the analyzed image file.
- *INTERQUARTILE\_DENSITY*. The interquartile range (IQR) of the raw signal intensities.
- *RATIO\_RANGE*. The ratio range for two-color microarray applications. Ratio range is calculated by dividing the array into a grid of uniformity blocks (4 x 18 on 2.1M arrays and 4 x 6 on 385K, 4x72K or 12x135K arrays), calculating the average  $\log_2$  ratio of each block and calculating the range from the block averages.



This metric applies only to two-color applications.

- *SIGNAL\_RANGE*. The signal range represents the signal range on a per channel basis for both one- and two-color microarray applications. Similar to ratio range, the signal range is calculated based on the range of signal means from the average signal per block.
- *UNIFORMITY\_MEAN*. The mean signal intensity of all the probes in each uniformity block.
- *UNIFORMITY\_CV*. The coefficient of variation of the block uniformity means.
- *NUM\_EMPTY*. The number of empty features present on the array.
- *MEAN\_EMPTY*. The mean signal intensity of empty features present on the array.
- *NUM\_EXPERIMENTAL*. The number of experimental features present on the array.
- *MEAN\_EXPERIMENTAL*. The mean signal intensity of the experimental features present on the array.
- *NUM\_RANDOM*. The number of random control features present on the array.
- *MEAN\_RANDOM*. The mean signal intensity of the random control features present on the array. These probes generally have the same length and GC characteristics as the experimental probes on the array, and can be used to estimate the amount of non-specific binding in the hybridization.
- *LHC\_DEL*. The mean  $\log_2$  ratio of the probes corresponding to the deleted region of the LHC-2 DNA.



Disregard the LHC and SLOPE metrics provided on the report. LHC controls are not used in NimbleGen gene expression analysis.

## Step 6. (Optional) Create Pair Reports

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

Pair reports contain the raw data, listing the probe intensities of the array. Create .pair files for all arrays to be analyzed together.

1. Select Analysis -> Reports -> Pair. Refer to the *NimbleScan Software User's Guide* for further details on Pair reports.
2. To choose the gridded files to analyze, click Add images.
3. Navigate to the directory containing your array image files (.tif) and select the file(s).
4. Click Add images.
5. Browse to select the correct design file (.ndf) in the Design File disk provided with your array for the *Design file* field.
6. Browse to select the desired output file destination in the *Choose the destination folder* field.
7. Click Report. NimbleScan software creates one Pair report (.pair) for each image file.

## Step 7. Analyze Data

NimbleScan software normalizes expression data using quantile normalization as described by Bolstad, et al. (*Bioinformatics* 2003; 19:185).

Gene calls are generated using the Robust Multichip Average (RMA) algorithm as described by Irizarry, et al. (*Nucleic Acids Res.* 2003; 31:e15 and *Biostatistics* 2003; 4:249).

1. Select Analysis -> Expression -> RMA Analysis.  
 RMA analysis must be done with arrays that were made with the same design file.
2. In *Step 1: Choose the data files to process...*, click Add files to add all the .pair files generated earlier in Step 6 or the gridded .tif files generated earlier in Step 3. All technical replicates (same labeled sample analyzed on arrays made with same design) should be normalized together.
3. In *Step 2: Choose the desired algorithm settings...*, leave the default selections in these fields:
  - *Background correction?* field
  - *Normalize?* field
  - *Save normalized pair data?* field
  - *By container* fieldRefer to the *NimbleScan Software User's Guide* to customize settings.
4. In *Step 3: Choose the destination folder...*, select the desired destination folder for the analyzed data. Roche NimbleGen recommends you create a new folder for each experiment.
5. In *Step 4: Run RMA...*, click Run RMA. It may take a few minutes to complete the analysis. The page will return to blank when the analysis is complete.

- 6.** Go to the destination folder that contains the analyzed data and review the files:

File Format	Content	Viewing and Analysis Method
Calls File (_RMA.calls)	Normalized gene expression value and accession number for each gene interrogated by the array	Spreadsheet software (such Microsoft Excel) or ArrayStar or similar microarray gene expression analysis software
Pair File (_norm_RMA.pair)	Identifying information about each probe, their location within the gene, and signal intensity of each probe	Microsoft WordPad



To generate Non-normalized Calls reports to be used for normalization using different software, select **Analysis -> Reports -> Gene Call** in NimbleScan software.

The .pair and .calls files are compatible with a number of commercially available expression analysis software tools that can be used for further analysis of differentially expressed genes. Go to [www.nimblegen.com/products/software/](http://www.nimblegen.com/products/software/) for more information.

## Notes

# Chapter 7. Troubleshooting

This chapter helps you troubleshoot problems that occurred with your microarray experiment.

## Sample Quality

Problem	Possible Cause	Recommended Corrective Action
<b>260/230 Absorption Ratio is less than 1.8.</b>	Sample is contaminated with carbohydrate or phenol/chloroform.	Clean up samples using a cleanup column.
<b>260/280 Absorption Ratio is less than 1.8.</b>	Sample is contaminated with protein.	Clean up samples using a cleanup column.
<b>260/280 Absorption Ratio is greater than 2.0.</b>	Sample is contaminated or degraded.	Clean up samples using a cleanup column. Or if degraded, repeat sample extraction.

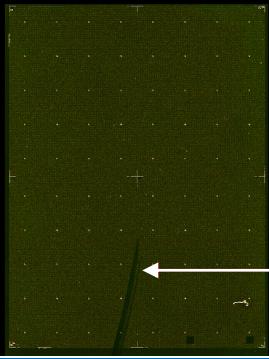
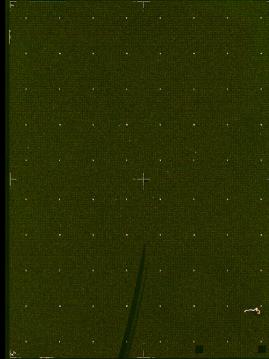
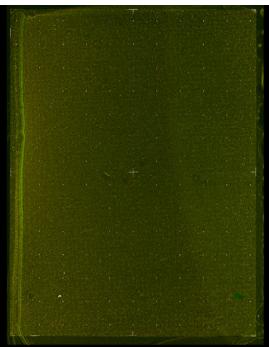
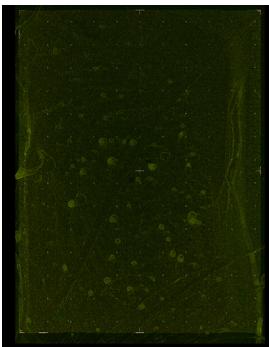
## Labeling

Problem	Possible Cause(s)	Recommended Corrective Action
<b>Labeling yield is less than 6 µg per reaction.</b>	Sample is contaminated or degraded.	Check absorption ratios and gel image. If necessary, clean up samples or repeat sample extraction. Repeat labeling.
	Primers were not diluted correctly. β-Mercaptoethanol was not added. Diluted primers are older than 4 months.	Prepare a fresh dilution of nonamer primers, ensuring that the β -Mercaptoethanol is fresh (opened less than 6 months). Repeat labeling.
	Klenow enzyme is expired or degraded.	Check the expiration date and follow the labeling kit's storage requirements. Repeat labeling, using fresh enzyme, if necessary.
	Primers are degraded.	Store primers at -20°C, protected from light, and avoid freeze-thaw cycles. Repeat labeling, using fresh primers, if necessary.
	dNTPs are expired or degraded.	Check the expiration date, follow the labeling kit's storage requirements, and avoid freeze-thaw cycles. Repeat labeling, using fresh dNTPs, if necessary.

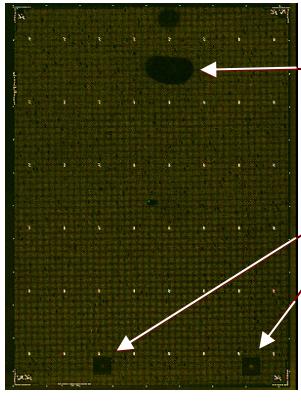
## Hybridization

Problem	Possible Cause	Recommended Corrective Action
<b>Mixer is poorly aligned on slide.</b>	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.
<b>Hybridization solution does not enter the hybridization chamber.</b>	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.
<b>Bubbles formed when loading the sample into the mixer's hybridization chamber.</b>	Air was present in the pipette tip.	<p>Use Gilson Positive Displacement Pipettes and follow the instructions on page 27 for proper loading technique.</p> <p>Using the pipette, remove the bubbles and replace with hybridization solution.</p> <p>Remove the bubbles or push them to the corners using the mixer brayer.</p>
<b>Sample leaked out of the mixer before or during the hybridization.</b>	<p>The mixer was not fully adhered to the slide due to incomplete braying.</p> <p>The mixer port seals/multi-port seals were not fully adhered to the mixer.</p>	<p>Refer to page 26 for proper braying technique. Repeat hybridization.</p> <p>Refer to page 27 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.</p>

## Scanning

Problem	Possible Cause	Recommended Corrective Action
<b>Scratches and/or fingerprints are seen on the array image.</b>	The slide was mishandled or dropped.	Grip the slide only on its edges and handle with care. Wear gloves when handling slides.
 Scratch		
<b>Dust is seen on the array image.</b>	The array was exposed to environmental dust.	If the amount of dust present is small, use a compressed gas nozzle to gently blow compressed nitrogen or argon gas across the array to remove the dust. Rescan the array.  If the amount of dust is excessive, repeat all wash steps, dry, and scan.
 Dust		
	Dust or dirt was present in the microarray dryer.	Clean the NimbleGen Microarray Dryer or other microarray dryer as instructed in its operator's manual. Repeat all wash steps, dry, and scan.
<b>Wash artifacts are seen on the array image.</b>	Slides were not washed and dried completely.	Use the recommended microarray dryer: the NimbleGen Microarray Dryer.  Repeat the slide wash, dry, and scan steps, making sure to transfer the slide immediately from the wash solution to the microarray dryer. Blot residual wash buffer from the edges of the slide. Make fresh wash buffers for each batch of slides.
		

Problem	Possible Cause	Recommended Corrective Action
<b>Bright streaks are seen on the array image.</b>	The microarray dryer does not accelerate fast enough.	Ensure proper function and maintenance of the microarray dryer. The microarray dryer should achieve a top speed of at least 1,400 rpm in a minimum of 0.8 seconds.
<b>Part of the array is missing from the array image.</b>	The scan area is not specified properly.	Refer to page 37 for instructions on how to specify the scan area. Repeat the scan ensuring that fiducial features are included in the scan area.
<b>The array image appears dim.</b>	Gain or PMT settings are not adjusted correctly.  Hybridization and/or wash conditions are too stringent.	Refer to the scanner documentation (or page 39 of this <i>User's Guide</i> if using the MS 200 scanner) for instructions on how to adjust gain or PMT settings. Repeat scan.  Check that the hybridization solution was prepared correctly (refer to page 24) and the NimbleGen Hybridization System is set to and maintaining 42°C. Repeat hybridization.
		Refer to page 29 for proper washing technique. Repeat hybridization.
	Sample leaked out of the mixer during hybridization due to incomplete braying.  Cy dye is degraded due to exposure to light, ozone, and/or humidity.	Refer to page 26 for proper braying technique. Repeat hybridization.  Store primers at -20°C, protected from light. Maintain ozone levels below 20 ppb and humidity levels below 40%. Repeat hybridization.
<b>The fiducial features appear dim or blank.</b>	The alignment oligo was either not added to the hybridization solution or was degraded due to repeated freeze-thaw cycles.	Repeat hybridization, using fresh alignment oligo, if necessary.

Problem	Possible Cause	Recommended Corrective Action
<b>Features appear blank on portions of the array.</b>	The slide contains a scratch or fingerprint.	Grip the slide only on its edges and handle with care. Wear gloves when handling slides. Repeat hybridization.
	One or more bubbles were present in the hybridization chamber.	Repeat hybridization if blank regions cover greater than 5% of the array area.
	 <p>A microarray image showing a dark grid of features. A large, irregularly shaped dark spot is labeled "Bubble". Two smaller arrows point to specific features on the array, labeled "NimbleGen Control Regions".</p>	
<b>The array image is too bright.</b>	Wash buffer dried onto the array surface in between wash steps.	Ensure that slides are transferred quickly between wash steps. Repeat hybridization.
	Hybridization and/or wash conditions are not stringent enough.	Check that the hybridization solution was prepared correctly (refer to page 24) and the NimbleGen Hybridization System is set to and maintaining 42°C. Repeat hybridization. Refer to page 29 for proper washing technique. Repeat hybridization.
	Gain or PMT settings are not adjusted correctly.	Refer to the scanner's documentation (or page 39 of this <i>User's Guide</i> if using the MS 200 scanner) for instructions on how to adjust PMT settings. Repeat scan.

Problem	Possible Cause	Recommended Corrective Action
<b>The array image brightness is uneven.</b>	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 <i>NimbleGen Hybridization System User's Guide</i> for troubleshooting information.
	Sample leaked out of the mixer during hybridization due to incomplete braying	Refer to page 26 for proper braying technique. Repeat hybridization.
	The sample pellet was not properly rehydrated or mixed with the hybridization solution.	During sample preparation, be sure to vortex and spin the sample before and after the 95°C incubation. Repeat hybridization.
	Array washing was not done properly.	Refer to page 29 for proper washing technique. Repeat hybridization.
<b>Features appear out of alignment.</b>	Image has not been gridded.	Standard procedure is to grid the image using NimbleScan software.

## Sample Tracking Controls (STCs)

Problem	Possible Cause	Recommended Corrective Action
<b>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.</b>	Each sample was not resuspended in an STC, or the STC was degraded from repeated freeze-thaw cycles.	Repeat hybridization, using a fresh STC, if necessary.
<b>STC features representing multiple STCs are visible on the array image or reported in the Sample Tracking report.</b>	Sample integrity was compromised during sample preparation, loading, or hybridization.	<p>Repeat hybridization, ensuring the following:</p> <ul style="list-style-type: none"> <li>■ The mixer is fully adhered to the slide before loading sample. Refer to page 26 for proper braying technique.</li> <li>■ Excess sample is removed from around the loading ports. Refer to page 27 for proper sealing technique.</li> <li>■ The mixer port seals/multi-port seals were not fully adhered to the mixer after loading sample. Refer to page 27 for proper sealing technique.</li> </ul>

## Data Analysis

Problem	Possible Cause	Recommended Corrective Action
<b>NimbleScan fails to open the image TIFF file or Multiplex images fail to burst.</b>	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.
<b>Multiplex images were burst incorrectly.</b>	NimbleScan software reads only 16-bit grayscale images.	Refer to page 37 for instructions on how to specify the scan area. Check the image using the scanner software and rescan.
	The entire array area was not scanned.	Refer to page 37 for instructions on how to specify the scan area. Crop the image in NimbleScan software and attempt bursting again.
	The scanned area is too large.	Refer to page 37 for instructions on how to specify the scan area. Crop the image in NimbleScan software and attempt bursting again.
<b>The auto align function fails to grid the array image.</b>	The array area is not centered in the scanned image.	Refer to page 37 for instructions on how to specify the scan area. Crop the image in NimbleScan software or rescan if necessary.
	Fiducial features are dim.	Perform a manual alignment as described in the <i>NimbleScan User's Guide</i> .
	NimbleScan v2.4 or later was not used.	Install the latest version of NimbleScan software. Reload the image and perform the auto align function.
<b>The auto align function improperly grids the array.</b>	Bright artifacts are present in the corners of the array image.	Perform a manual alignment as described in the <i>NimbleScan User's Guide</i> .

# Appendix A. Expression Data Analysis

You can review the resultant pair and calls files from an RMA analysis and analyze the expression profile of each sample. This appendix briefly describes how to use the ArrayStar software to import normalized data and perform a variety of analytical and visualization techniques.

## Step 1. Install the Necessary Software

Download a free, fully functional trial version of the DNAStar ArrayStar software at [http://nimblegen.dnastar.com/forms/demo\\_requestnimble.php](http://nimblegen.dnastar.com/forms/demo_requestnimble.php).

## Step 2. Import Your Expression Data

1. Open ArrayStar software.
2. To import .calls files:
  - a. Select File -> Import Experiments.
  - b. Click Browse to find and select the .calls files to import.
3. To import gene annotations:
  - a. Select File -> Import Annotations.
  - b. Click Browse to find and select the .ngd files to import.
4. Select File -> Save Project to save your files as an ArrayStar project (.dmaproj).

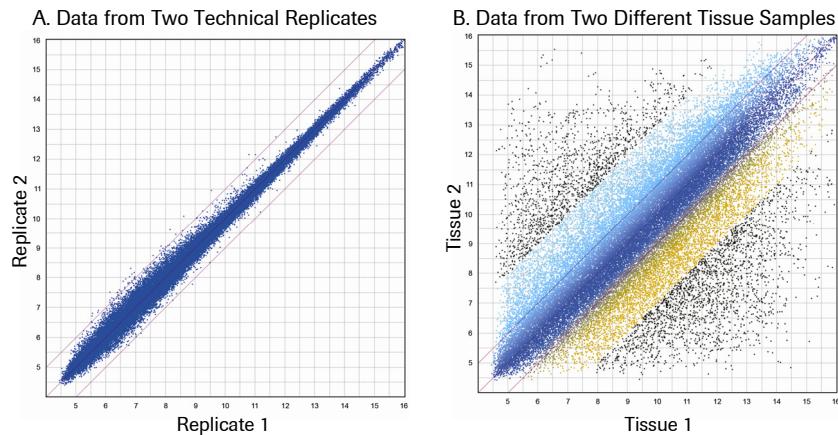
## Step 3. Analyze Your Expression Data

There are many techniques for analyzing microarray expression data from your ArrayStar project. Following is a description of some options supported by the ArrayStar software.

1. To view a pair-wise comparison in the Scatter Plot view:
  - a. Select Graphs -> Scatter Plot.

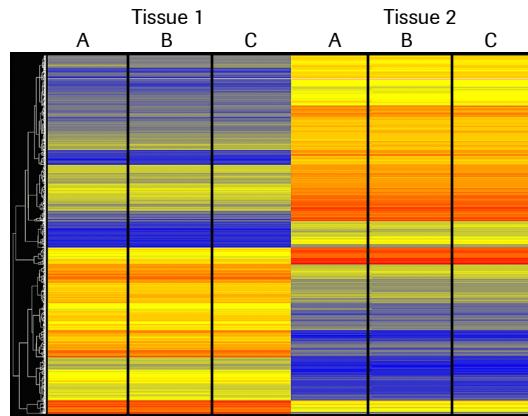
Figure 27 shows example scatter plots for data from two technical replicates (panel A) and for data from two different tissue samples (panel B). The statistics panel from the Scatter Plot Info Pane provides information on the correlation between the samples ( $r^2$ ) and the number of genes present at specified fold changes between samples.

- b. Click the link for a specific fold change to select the genes for further analysis.



**Figure 27: Scatter Plots**

2. To view a Hierarchical clustering of your data set in the Heat Map view:
  - a. Select Clustering -> Hierarchical.  
Figure 28 shows an example heat map for the genes expressed at  $\geq 8$ -fold difference between the samples shown in Figure 27B.
  - b. Select genes of interest by clicking on a node in Gene Tree, shown on the Y axis of the Heat Map, and then select Data -> Show Gene Table.



**Figure 28: Heat Map**

3. To cluster the selected genes with the k-Means method:
  - a. Select Clustering -> k-Means.
  - b. Display your results in the Line Graph Thumbnails view.
  - c. Double-click a graph in the Line Graph Thumbnails view to launch the Line Graph view for the cluster you selected.

- 4.** To view detail information for each gene:
  - a.** Select **Data -> Show Gene Table**.
  - b.** Add columns to the table using the buttons in the Gene Table toolbar.
- 5.** Select **File -> Save Project** to save your work.

## Notes

---

---

---

---

---

---

---

---

---

---

---

---

# **Appendix B. Limited Warranty**

## **ROCHE NIMBLEGEN, INC. NIMBLEGEN ARRAYS**

### **1. Limited Warranty**

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

B. Under no circumstances shall Roche NimbleGen’s liability to Customer exceed the amount paid by Customer for the Services and Products to Roche NimbleGen. Roche NimbleGen will bear all reasonable shipping costs if service is re-performed at Roche NimbleGen or the Products are replaced. This warranty does not apply to any defect or nonconformance caused by (i) the failure by Customer to provide a suitable storage, use, or operating environment for the Materials or Customer’s submission of substandard quality Materials or contaminated or degraded Materials to Roche NimbleGen, (ii) Customer’s use of non-recommended reagents, (iii) Customer’s use of the Products, Materials or Data for a purpose or in a manner other than that for which they were designed, (iv) the failure by Customer to follow Roche NimbleGen’s published protocols; or (v) as a result of any other abuse, misuse or neglect of the Products, Materials or Data by Customer. This warranty applies only to Customer and not to third parties.

C. TO THE FULLEST EXTENT PERMITTED BY APPLICABLE LAW, ROCHE NIMBLEGEN DISCLAIMS ALL OTHER REPRESENTATIONS, AND WARRANTIES, EXPRESS OR IMPLIED, WITH RESPECT TO THE PRODUCTS, SERVICES AND DATA, INCLUDING BUT NOT LIMITED TO, ANY IMPLIED WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE OR NON-INFRINGEMENT. CUSTOMER’S SOLE REMEDY FOR BREACH OF WARRANTY IS STATED ABOVE.

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

## **2. FURTHER LIABILITY LIMITATION**

TO THE FULLEST EXTENT PERMITTED UNDER APPLICABLE LAW, ROCHE NIMBLEGEN SHALL NOT HAVE ANY LIABILITY FOR INCIDENTAL, COMPENSATORY, PUNITIVE, CONSEQUENTIAL, INDIRECT, SPECIAL OR OTHER SIMILAR DAMAGES, HOWEVER CAUSED AND REGARDLESS OF FORM OF ACTION WHETHER IN CONTRACT, TORT (INCLUDING NEGLIGENCE), STRICT PRODUCT LIABILITY OR OTHERWISE, EVEN IF ROCHE NIMBLEGEN HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. CUSTOMER UNDERSTANDS THAT ANY RISKS OF LOSS HEREUNDER ARE REFLECTED IN THE PRICE OF THE SERVICES AND PRODUCTS AND THAT THESE TERMS WOULD HAVE BEEN DIFFERENT IF THERE HAD BEEN A DIFFERENT ALLOCATION OF RISK.

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

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

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

NIMBLEGEN is a trademark of Roche. Other brands or product names are trademarks of their respective holders.

*Published by:*  
Roche NimbleGen, Inc  
504 S. Rosa Road  
Madison, WI 53719  
USA

[www.nimblegen.com](http://www.nimblegen.com)

© 2010 Roche NimbleGen, Inc.  
All rights reserved.